CHARACTERIZING THE PROTECTION OF AN N-TERMINAL ACTIVE CORE PEPTIDE WITHIN β-AMYLOID AGAINST β-AMYLOID NEUROTOXICITY

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia in the aging population. As the disease progresses, AD leads to cognitive deficits affecting memory, changes in personality, and language dysfunction. AD is characterized by the pathological extracellular accumulation of fibril β-amyloid (Aβ) into senile plaques and the intraneuronal accumulation of the microtubule-associated protein tau as neurofibrillary tangles. Initially, insoluble fibrillary Aβ was believed to be central to disease pathogenesis, but more recent evidence implicates soluble oligomeric Aβ as the trigger behind the earliest cognitive deficits in AD. Under normal conditions, Aβ at low, physiological levels (pM) functions as a positive neuromodulator, enhancing synaptic plasticity and function. In addition, mouse models lacking Aβ show deficits in cognitive function and loss of synapses, suggesting, therefore, a vital role of Aβ in the maintenance of synaptic activity. On the other hand, pathological levels of Aβ (high nM-μM) cause irreversible degeneration of neuronal processes and the loss of synaptic function and connections in select areas of the brain. Previously, we reported that an endogenous N-terminal fragment derived from full-length Aβ retains the latter's positive neuromodulatory activity and, notably, protects against Aβ-induced synaptic and memory deficits. Furthermore, through subsequent mutational analysis, we found a core sequence (YEVHHQ: N-Aβcore) within the N-terminal fragment accounting for its activity. Here, we aimed to characterize the neuroprotective potential of the N-Aβcore against Aβ-induced neuronal and synaptic damage, while elucidating the neuroprotective mechanism(s) of the N-Aβcore.

Utilizing a neuronal toxicity model (rodent hybrid neuroblastoma cells transfected with $\alpha4\beta2$ nicotinic acetylcholine receptors, nAChRs), the N-Aβcore was shown to retain receptor-linked activity. Subsequent mutational analysis demonstrated that the two histidine residues, and to a lesser degree, the tyrosine residue in the N-Aβcore are essential for this activity. In comparison to the sustained-elevated Ca^{2+} response elicited by Aβ, the N-Aβcore and N-terminal fragment displayed differential Ca^{2+} responses, suggesting the activation of an alternative, Aβ-independent pathway. In addition, the N-Aβcore was shown to be neuroprotective against Aβ-induced oxidative stress, ER stress, mitochondrial dysregulation, apoptosis, and synaptic dysfunction.

We have also previously reported an increase in activity of various mitogen-activated protein kinases (MAPKs) in response to prolonged, sustained Aβ exposure. We therefore addressed whether the N-Aβcore has an impact on Aβ-linked MAPK activation. In our neuronal toxicity model, the N-Aβcore reduced the Aβ-induced MAPK activity. Interestingly, preliminary evidence indicates that low levels (pM) of the N-Aβcore nearly abolished c-Jun N-terminal kinase (JNK) activity. Furthermore, low levels of the N-Aβcore showed an increase in cAMP response element-binding protein (CREB) activity and expression, which has been shown to be downregulated in AD and AD models. Taken together, these findings suggest that the N-Aβcore is neuroprotective against Aβ-induced neuronal and synaptic toxicity by partially inhibiting Aβ binding to target receptors and subsequently activating an Aβ-independent neuroprotective pathway.

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ABAD	Aβ-binding alcohol dehydrogenase
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer Disease
AICD	APP intracellular domain
AKT	Protein-kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APLP1	APP-like protein 1
APLP2	APP-like protein 2
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
ARE	Antioxidant response element
Aβ	Amyloid Beta
BACE1	Beta-site APP cleaving enzyme 1
BBB	Blood Brain Barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
CA1	Cortical area 1
Ca^{2+}	Calcium
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CFC	Contextual fear conditioning
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CREB	Cyclic AMP response element binding protein
CSF	Cerebrospinal fluid

CTFα	Carboxy terminal fragment of APP after α-cleavage
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated protein
fEPSP	Field excitatory postsynaptic potential
GPx	Glutathione peroxidase
HFS	High frequency stimulation
IL-1β	Interleukin 1β
IP_3	Inositol triphosphate
JNKs	c-Jun N-terminal kinases
K^+	Potassium
LFS	Low frequency stimulation
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR5	Metabotropic glutamate receptor 5
N-Aβ fragment	N-terminal Aβ fragment, $Aβ_{1-15}$
N-Aβcore	N-terminal Aβ core sequence, $Aβ_{10-15}$
Na^+	Sodium
NAC	N-acetylcysteine
nAChRs	Nicotinic acetylcholine receptors
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB

NFAT	Nuclear factor of activated T cells
NGF	Nerve growth factor
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NrF2	Nuclear factor E2-related factor 2
PBS	Phosphate buffered saline
PERK	Protein kinase-like endoplasmic reticulum kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PI3-kinase	Phosphoinositide 3-kinase
PirB	Paired immunoglobulin-like receptor B
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma
pM	Picomolar
PP1	Protein phosphatase 1
PPARγ	Peroxisome proliferator-activated receptor gamma
PrPc	Cellular prion protein
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAPKs	Stress-activated protein kinases
sAPPα	Soluble APP-alpha

sAPPβ	Soluble APP-beta
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
sGC	Soluble guanylyl cyclase
SIRT1	Sirtuin family member 1
SODs	Superoxide dismutases
TBS	Tri-buffered saline
TM	Transmembrane
TMRE	Tetramethyl rhodamine ethyl ester
TNF-α	Tumor necrosis factor α
TrkB	Tropomyosin receptor kinase receptor B
TTX	Tetrodotoxin
TUNEL	Terminal-deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UPR	Unfolded protein response
Wnt	Wingless-related integration site
μM	Micromolar

CHAPTER 1: INTRODUCTION

1.1 Alzheimer Disease

Alzheimer's disease (AD) is a neurodegenerative disease, currently incurable, which presents one of the largest unmet needs in medicine [1, 2]. AD is characterized by the accumulation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau and extracellular amyloid beta (Aβ) deposits evident as senile plaques [3]. With the progression of these pathological hallmarks, neuronal loss in select regions of the brain parallel the clinical symptom of progressive and gradual decline in cognitive function.

There are a number of existing hypotheses that have been proposed to explain the origins of AD, but the amyloid cascade hypothesis, while controversial, has been the most influential theory for research and drug development to date, suggesting that the formation, aggregation and deposition of Aβ peptides are primary events in AD pathogenesis [4–6]. This hypothesis gained ground when several key studies revealed that accumulation of soluble, oligomeric Aβ preceded accumulation of hyperphosphorylated tau, with the former being relatively specific for AD independent of plaque formation, and the latter being found in other disorders. More importantly, subsequent studies indicated that oligomeric Aβ may foster tau hyperphosphorylation in AD, with intracellular hyperphosphorylated tau being the primary driver of neuronal toxicity and, ultimately, neurodegeneration [7–9]. In addition, the discovery that AD could be inherited in an autosomal dominant fashion [10–12] further strengthened the amyloid cascade hypothesis claim. The autosomal mutations reside in genes coding for the amyloid precursor protein (APP), an integral type-1 transmembrane protein that is

sequentially cleaved by specific processing enzymes, known as secretases, or in genes coding for key components of one of secretases, namely presenilin 1 (PSEN1) [12] or PSEN2 [13] in the γ-secretase complex [14]. Cleavage by the β- and γ-secretases yields Aβ, while cleavage by α-secretase and γ-secretase (and/or β-secretase) is nonamyloidogenic [15–17]. Lastly, the most prominent genetic risk factor for late-onset AD, ApoE-ε4, was recently shown to drive amyloid pathology at the earliest (seeding) stage [18, 19].

In considering alternative models for the origin of the disease, it has been argued that extant evidence does not fully explain or fit easily with the simplest version of the amyloid hypothesis. One of the main objections to the hypothesis is that the amyloid load in the brain does not invariably correlate with the degree of cognitive impairments in patients, as PET imaging have revealed substantial amyloid burden (as aggregates or plaques) in a subset of individuals that do not manifest clinical symptoms. Moreover, treatments (e.g., immunization) that reduced amyloid burden did not lead to clinical improvement. Finally, neurodegeneration correlates best with neurofibrillary tangle burden. However, there remains a considerable body of evidence demonstrating that soluble Aβ oligomers, not monomers or insoluble fibrils or plaques, are responsible for synaptic dysfunction, neuronal damage and memory deficits exhibited in AD [20–22].

The overproduction and self-association of Aβ monomers into soluble oligomers and/or inadequate clearance of Aβ [23] leads to their accumulation, which increases oxidative stress [24], elicits mitochondrial dysfunction [25, 26], elevates presynaptic calcium [27], causes synaptic dysfunction [28, 29] and synaptic loss [30], and ultimately neuronal death [31] (Fig. 1). On the other hand, physiological levels of Aβ in healthy

normal brains have been found to enhance synaptic plasticity [32, 33], synaptic function [34–36] and fear memory [32], suggesting that Aβ acts as a positive neuromodulator at low concentrations (pM range). The prominent role Aβ plays in AD pathology makes it a reasonable target for therapeutic intervention(s), and the majority of drug research and development to date has been centered around the amyloid cascade hypothesis, aiming to reduce Aβ formation and aggregation, inhibiting Aβ toxicity and/or increasing Aβ clearance.

1.2 Amyloid Precursor Protein (APP) Processing

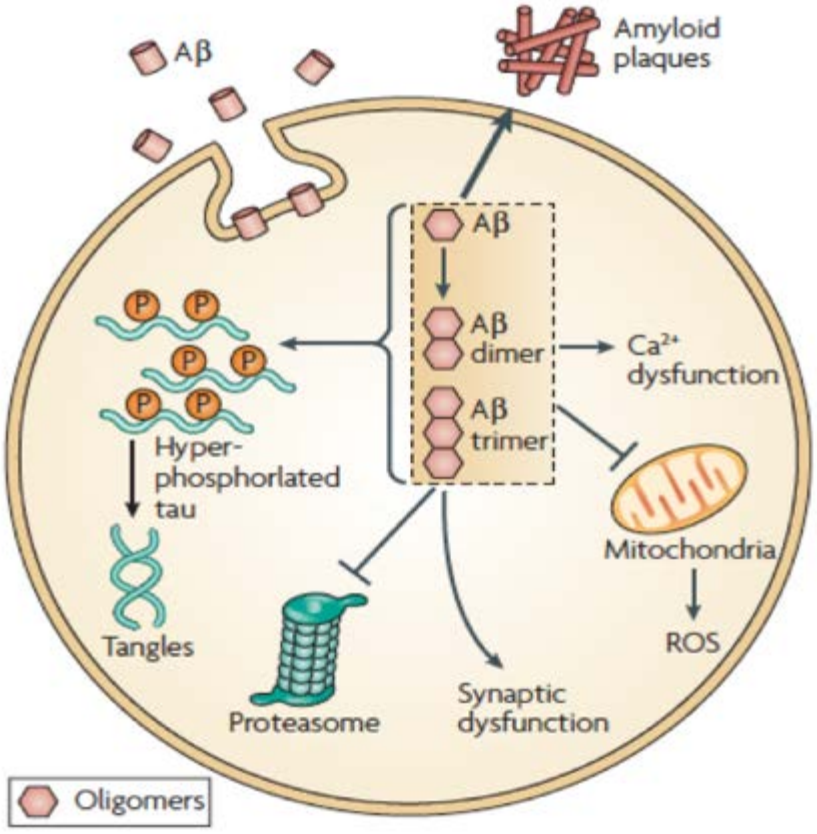


Figure 1: Aβ Toxicity

Accumulation of soluble Aβ oligomers leads to Ca^{2+} dysregulation and dysfunction, mitochondrial dysfunction, oxidative stress, hyperphosphorylation of tau, proteasome disruption and synaptic deficits. (Figure adapted from [31])

The amyloid precursor protein (APP) is a single-spanning transmembrane protein that is abundantly expressed in the brain and located on chromosome 21 [37] with three major isoforms arising from alternative splicing [11]. APP belongs to a protein family that consists of APP-like protein 1 (APLP1) and 2 (APLP2) in mammals [38, 39], but the Aβ domain is unique to APP. Unfortunately, the function of APP is still not widely understood, but it has been suggested to be involved in neurite outgrowth and synaptogenesis [40, 41], neuronal protein trafficking [42], transmembrane receptor signaling [43, 44], calcium signaling [45], cellular and synaptic adhesion [46] and synaptic and neuro-protection [40, 47–49]. More importantly, APP has gained notoriety from its central role in Aβ production and AD pathology.

APP is produced in large quantities in neurons and is delivered to the axon where it is then transported to the synaptic terminals [50]. It undergoes regulated sequential cleavage of either α- (nonamyloidogenic pathway) or β- (amyloidogenic pathway) secretase followed by γ-secretase. The nonamyloidogenic pathway involves the cleavage of APP within the Aβ domain by an α-secretase, releasing a soluble ectodomain of APP called sAPPα. Studies have shown sAPPα play a critical role in neuronal plasticity and survival, and be protective against excitotoxicity [51, 52]. sAPPα also regulates neural stem cell proliferation and is important for early central nervous system (CNS) development [53, 54]. Interestingly, sAPPα has also been shown to be able to rescue APP deficient mice abnormalities [55], suggesting that most of the physiological role of APP is mediated by sAPPα. After α-cleavage (CTFα), the carboxy terminal fragment of APP remains in the membrane and is further cleaved by γ-secretase, releasing a P3 fragment, which is rapidly degraded and is believed to be of no importance, and an APP

intracellular domain (AICD). The AICD is believed to regulate the transcription of neprilysin (an Aβ degradation enzyme), BACE1 (a β-secretase) and APP [56, 57].

In a mutually exclusive pathway, the production of Aβ is through the sequential enzymatic cleavage via the β- and γ-secretases [58] (Fig. 2). BACE1, the major β-secretase involved in APP metabolism, is a transmembrane aspartic protease that cleaves at the β-sites of Aβ (Asp1 and Glu11), releasing an sAPPβ ectodomain. The CTFβ is cleaved by γ-secretase at one of several sites varying from +40 to +44 to generate various Aβ peptides. As Aβ is partially derived from the transmembrane (TM) domain of APP, the peptide has a C-terminal hydrophobic domain, which has been shown to largely but not exclusively account for its aggregation and neurotoxicity [59, 60].

1.2.1 The N-Aβ fragment ($Aβ_{1-15}$)

In contrast, a larger hydrophilic domain resides on the N-terminal side of the peptide. It has been shown that a 15-16 amino acid N-terminal Aβ fragment (N-Aβ fragment) can be produced from this domain via an alternative α-secretase-linked pathway and is present at significant levels in cerebrospinal fluid (CSF) [15] (Fig. 2). Due to its hydrophilicity, the N-Aβ fragment does not form oligomers and is non-toxic [61]. Moreover, pM-nM concentrations of the N-Aβ fragment are approximately twice as effective as Aβ in stimulating receptor-linked increases in Ca^{2+}, enhancing long-term potentiation (LTP) and enhancing contextual fear conditioning (CFC)[62].

1.2.2 The N-Aβcore ($Aβ_{10-15}$)

The N-Aβcore is not endogenously produced (Fig. 2) but is believed to be the responsible core sequence for Aβ's and the N-Aβ fragment's receptor-linked Ca^{2+} responses [62] and thus potentially accounting for the neuromodulatory activity of Aβ. The small size and non-toxic nature of the N-Aβcore makes it an ideal scaffold for therapeutic exploration and development.

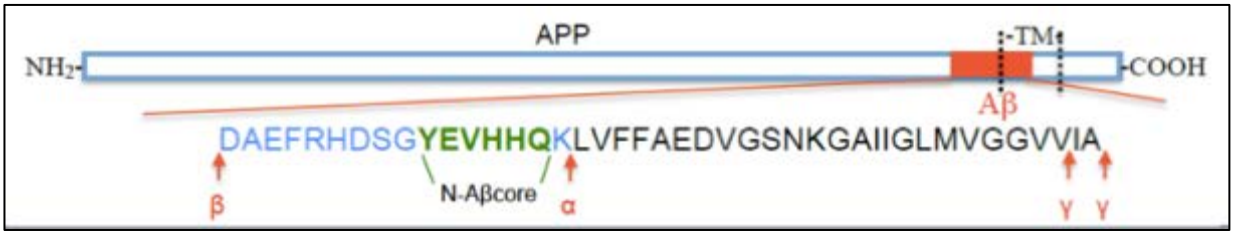


Figure 2: APP Processing

APP is a transmembrane spanning protein. The production of Aβ is through the sequential cleavage of β- and γ- secretase giving rise to varying lengths of Aβ. The N-terminal Aβ fragment (shown in blue) is produced through a lesser known alternative pathway with cleavage by the β- and α- secretase. The lysine residue is cleaved off by a C-terminal carboxypeptidase, thus producing a 15aa fragment. The N-Aβcore (shown in green) is not produced endogenously.

1.3 Current Therapeutic Strategies Specific to Aβ Toxicity

There are three main intervention strategies for therapy aimed at Aβ: reducing Aβ production, facilitating Aβ clearance, and preventing Aβ aggregation.

Reducing Aβ production through inhibiting the activities of γ-secretase or β-secretase has been shown to reduce the levels of plasma, brain and CSF Aβ in multiple models overexpressing APP [63–68]. Although able to reduce Aβ, the γ-secretase inhibitors cause drug-related toxicities in the gastrointestinal tract [69], thymus [70] and spleen [65, 71]. As for β-secretase, small-molecules capable of penetrating the blood brain barrier (BBB) while potently inhibiting the enzyme in view of its large active site are under

development. On the other hand, vaccine-targeting or passive immunotherapy to lower Aβ has proven effective [72, 73] but without any clinical significant improvement and, worse, runs the risk of inflammatory - microhemorrhagic responses.

Interventions focused on the clearance of Aβ have been aimed at stimulating Aβ degradation [74–76], export of Aβ across the BBB [77–79], or removing peripheral Aβ [80]. Many of these drugs have been substantiated in animal models and are undergoing extensive clinical trials in humans, but have all failed to meet their primary clinical endpoints, namely improvement in memory and cognitive tasks.

Inhibition of Aβ aggregation has been attempted through the development of small-molecules interacting with Aβ during oligomerization into fibrillar forms [81], but also without clinical success.

Although much progress has been made in our understanding of AD pathophysiology, a large number of molecules developed for therapeutic application have fallen short in clinical trials. Therefore, a new therapeutic agent that not only alleviates the symptoms but also protects against further neuronal damage is greatly needed. Next, we will focus on the current array of neuroprotective agents reported to have identified activities against Aβ-induced neurotoxicity at various levels and will assess their therapeutic potential

1.3.1 Neuroprotection

Neuroprotection may result from endogenous factors or exogenous interventions mitigating metabolic, physiological or physical insult or damage in the nervous system at the cellular or subcellular level, potentially slowing or stopping the progression of an

underlying condition (e.g., stroke) or disease (e.g., AD) [82]. Agents that claim to have neuroprotective properties aim to prevent or slow down disease progression by slowing down, halting or reversing synaptic and/or neuronal dysfunction and/or loss as a means to preserve neuronal population survival. Many of the current neuroprotective treatments for neurodegeneration facilitate the reduction of oxidative stress through the application of antioxidants [83–87] and the block of excitotoxicity through glutamate antagonists [88–92]. Neuroprotective strategies specific for Aβ toxicity have likewise been focused on targeting Aβ-induced oxidative stress [86, 93] and excitotoxicity [94, 95]. Additional neuroprotective strategies include blocking Aβ interactions with high affinity targets [96, 97], down-regulating stress kinase signaling cascades [98, 99], blocking activation of caspases [100] and upregulating anti-apoptotic pathways [101–105]. Here, we will take a closer look at the most common, targeted neuroprotective strategies against Aβ toxicity.

1.3.2 Reducing Oxidative Stress

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and their removal by the antioxidant system by either an overproduction of ROS or a reduction in antioxidant activity [106, 107]. Oxidative stress arises in the early stages of AD [108, 109] and progresses with age [110]. Oxidative stress has been shown to correlate with increased Aβ deposition in the brain [111, 112], increased apoptosis through activation of stress signals and caspases [113, 114], and promotion of Aβ production through β- and γ-secretase activity [115, 116]. The addition of vitamin E has been shown to inhibit Aβ toxicity, protein oxidation, and stress kinases *in vitro* [114, 117], with reports of lowering risk for AD in humans [117, 118], though its efficacy has been

controversial. In addition, N-acetylcysteine (NAC), a potent antioxidant, may also protect against Aβ toxicity through induction of anti-apoptotic signaling pathways [119]. Although oxidative stress is a well-established pathology of AD, the use of antioxidants as neuroprotective therapeutics has yielded conflicting results, with no clear, direct link between the administration of vitamin E or NAC and AD incidence.

1.3.3 Preventing Excitotoxicity

Proper functioning of NMDA (N-methyl-D-aspartate) receptors is important for synaptogenesis, synaptic remodeling, and plasticity [120]. Aβ has been implicated in triggering excitotoxicity in AD through sustained activation of NMDA and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) -type glutamate receptors causing excessive intraneuronal Ca^{2+} levels due to excess glutamate resulting from oxidative stress and Aβ accumulation [121, 122]. Additionally, excess Aβ enhances sensitivity to glutamate-induced excitotoxicity [123, 124] by inhibiting glutamate re-uptake [125, 126]. Current therapies to protect against Aβ-induced excitotoxicity include melatonin [127], Aβ monomers [128] and memantine [95]. The hormone melatonin is a free radical scavenger, reducing oxidative stress by removing singlet oxygen, superoxide anion radical, hydroperoxide, hydroxyl radical and the lipid peroxide radical [129]. Memantine is an NMDA channel antagonist, currently, as noted, in clinical use, that blocks the opening of the channel without inhibiting the physiological activation needed for LTP. Although modulating NMDA receptors to reduce glutamate-induced excitotoxicity by memantine improves patient behavior, cognition and function [130], it appears that

memantine largely alleviates symptoms without actually providing long-term protection against the underlying mechanistic cause of disease.

1.3.4 Modulating Stress-Activated Kinases

Stress-activated protein kinases (SAPKs) are a family of serine/threonine kinases involved in a variety of cellular responses, including cell proliferation and death. Activation of the c-Jun N-terminal kinases (JNKs) is associated with apoptosis [131, 132], while the inhibition of the JNK pathway may provide protection against neuronal death [133, 134]. Furthermore, prolonged sustained exposure to Aβ accumulation has been linked to JNK activation [113, 135, 136], which has, in turn, been associated with degenerating neurons in AD brains [135, 137]. Inhibiting the JNK pathway has become a strategic avenue to prevent apoptosis [138, 139], and a few JNK inhibitors/ modulators have entered clinical trials [98, 100, 140] but none for AD treatment. Although an attractive potential target to treating AD, inhibiting JNK activation (or any of the other SAPKs) systemically would likely have widespread adverse effects.

1.3.5 Neuroprotection by Blocking Aβ Targets

Aβ has been shown to interact with various receptor targets [141] to induce synaptic and neuronal dysfunction and death, the latter notably involving cellular prion protein (PrPc) [142] coupled to the metabotropic glutamate receptor 5 (mGluR5) [143], NMDA (and perhaps AMPA) receptors [144, 145] and nicotinic acetylcholine receptors (nAChRs) [61, 146](Fig. 3). The complement of receptors involved in Aβ-linked synaptic dysfunction, while partially overlapping with those connected to neuronal loss, appears to

involve a different array of targets with distinct temporal and spatial constraints whether the impact is on plasticity or synapse structure (i.e. dendritic spine morphology). While the development of compounds that can block Aβ interaction to various targets have been extensively explored, Aβ toxicity appears to be mediated by combined activation of Aβ targets, and thus identifying one agent that can prevent Aβ binding to all of the receptors has been challenging. Furthermore, compounds binding to these receptors and proteins may also affect their normal physiological functions.

In addition to competitive antagonism of Aβ to protect against neuronal toxicity, a number of receptor-based neuroprotective pathways have been identified and utilized as a potential strategy independent of Aβ. In AD models, the cyclic AMP response element binding protein (CREB), a constitutively expressed nuclear transcription factor that regulates neuronal survival and function, has been shown to be downregulated in hippocampal neurons [105]. It has been shown that CREB is important in neurotrophin-mediated signaling to neuronal survival [101] and neuroprotection via upregulation of Bcl-2 (B-cell lymphoma 2) transcription [147]. Contributing to a feed-forward mechanism, CREB is believed to upregulate neurotrophin expression and activity, importantly the brain-derived neurotrophic factor (BDNF) which is important in modulating synaptic plasticity [148] and a neurotrophin target receptor, the tropomyosin receptor kinase receptor B (TrkB) [149]. The use of neurotrophins, such as nerve growth factor (NGF) or BDNF, is an attractive alternative strategy for neuroprotection against Aβ toxicity, but to date, neurotrophins used for AD treatment have not been successful. Nonetheless, this approach remains promising.

A newly developed avenue has focused on Aβ itself to modulate endogenous regulatory activity rather than simply lowering Aβ levels. At low physiological levels (pM range) Aβ acts as a positive neuromodulator, as previously mentioned, and, interestingly, Aβ monomers were found to be neuroprotective via the PI3-kinase/Akt pathway through involvement of IGF-1/insulin receptors [128], resulting in downstream activation of CREB via phosphorylation [105]. While maintaining Aβ in monomeric form would be difficult if not impossible, a N-terminal fragment of Aβ (1-15/16) naturally derived from Aβ by the action of α-secretase, which does not oligomerize was shown to be responsible for the neuromodulatory activity of Aβ [62].

1.4 Neuroprotective Pathways

1.4.1 Inflammation

Chronic inflammation in the CNS has been shown to be associated with many neurodegenerative diseases, including AD [150, 151]. The two main factors believed to contribute to inflammation-induced neuronal death in AD are the increases in fibrillary Aβ deposits [152] and microglia activation [153, 154]. In the early stages of AD, low-levels of proinflammatory cytokines continuously act on microglia over a long period of time, and with the progression of the disease, elevated levels of Aβ exacerbate microglia activation, further increasing the expression of inflammatory genes [153].

Nuclear factor-κB (NF-κB) is a transcription factor involved in many biological processes including the regulation of cytokine production. Activation of NF-κB by TNF-α (tumor necrosis factor α), interleukin 1β (IL-1β) and lipopolysaccharide (LPS) via the canonical pathway or by LT(lymphotoxin)α/β, a CD40 ligand acting via the non-canonical

pathway [155] induces the production of inflammatory and oxidative stress related genes [156]. Interestingly, postmortem AD patient brain tissues show an increase in NF-κB activity in neurons and astrocytes surrounding Aβ plaques [157], and NF-κB is activated by Aβ in cultured neurons [158]. Targeting the reduction of NF-κB activity by inhibiting activation or DNA binding reduces microglia activation and subsequently the release of pro-inflammatory factors [159–161] resulting in cell survival. Unfortunately, inhibitors blocking NF-κB activity may consequently exacerbate the neurodegenerative processes by preventing the release of cytokines for neuroprotection, interfering with the critical physiological role of NF-κB.

1.4.2 Oxidative Stress

Oxidative stress is considered an essential factor in many neurodegenerative diseases and is believed to play an important mechanistic role in age-related degenerative processes. Increases in oxidative stress cause damage to lipids, DNA, proteins, mitochondrial damage and eventually cell death. Unfortunately, the brain is more sensitive than most other tissues to oxidative stress due to its low levels of protective enzymes such as glutathione peroxidases (GPxs), catalase [162] and superoxide dismutases (SODs) [163] underlying its inadequate ability to neutralize the ROS and RNS (reactive nitrogen species) [164]. Additionally, activated microglia contribute to oxidative stress in neurodegenerative disorders [165, 166]. It has also been shown that activation of Nrf2 in macrophages and microglia down-regulates NF-κB-induced inflammatory responses [167, 168].

The NrF2 signaling pathway plays a major role in protecting against oxidative stress [169, 170] by transcriptionally activating protective genes through the antioxidant response element (ARE) [171, 172]. AREs are promoter elements upstream of biotransformation phase II detoxifying enzymes and factors necessary for survival such as SODs [173], GPxs [174], catalase [175] and nicotinamide adenine dinucleotide phosphate (NADPH) [176]. Growing evidence has shown that the AD brain is under tremendous oxidative stress [110–112]. Notably, staining of hippocampal neurons in post-mortem AD patients showed a significant reduction of nuclear Nrf2 compared to normal cells, suggesting that Nrf2 was not being properly translocated into the nucleus [177], therefore Nrf2-mediated transcription was not being induced. Considering the evidence, studies targeting the Nrf2-ARE pathway for protection against oxidative stress-induced cellular death [178, 179] show promising results but the long-term neuroprotective effects still remain to be shown.

1.4.3 Mitochondrial Dysfunction

Mitochondria play important roles in cell survival and death by regulating energy metabolism and various death processes. Given their role in energy metabolism by oxidative phosphorylation, mitochondria convert 1-5% of the cellcular oxygen to ROS due to the leakage of electrons from the electron transport chain [180], making them one of the main producers of intracellular ROS under physiological conditions. Alternatively, under pathological conditions, the excess production and the cell's inability to neutralize ROS leads to oxidative stress-induced damage as previously mentioned. Mitochondrial dysfunction is one of the initial stages in apoptosis, and substantial evidence suggests

mitochondrial dysfunction as a key player in AD pathogenesis [181]. Taken further, some researchers have postulated that mitochondrial dysfunction is the primary event that triggers Aβ deposition, synaptic loss and dysfunction and tau neurofibrillary tangles [182].

The expression levels of SIRT1 (sirtuin family member 1), an NAD^+-dependent enzyme that regulates mitochondrial biogenesis [183], were shown to be significantly reduced in the parietal cortex of AD patients and negatively correlated with the accumulation of tau, suggesting that SIRT1 is associated with AD progression [184]. By contrast, the activation and overexpression of SIRT1 has been shown to be neuroprotective against neurodegenerative diseases [185, 186] by upregulating the peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1α), a regulator of mitochondrial biogenesis [187]. Various compounds have already been found to be neuroprotective through upregulating SIRT1 [188, 189].

The peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that controls many physiological and pathological processes [190], mainly lipid and glucose metabolism. Dysfunctional PPARγ has been implicated in many diseases of the CNS [191], and PPARγ agonists have been shown to ameliorate Aβ-induced learning and memory deficits [192, 193] as well as downregulate caspase 3 and 9 activity and upregulate PGC1α [194]. Increasing mitochondrial biogenesis by upregulating PGC1α through SIRT1 or PPARγ is a potential target to protect against mitochondrial dysfunction under pathological conditions.

While receptor-activated pathways have been linked to mitochondrial dysfunction, there is evidence that internalized Aβ binds with an Aβ-binding alcohol dehydrogenase (ABAD) [195] located in the mitochondria. ABAD plays an important role in metabolic

homeostasis and the Aβ-ABAD interaction affects this homeostasis and promotes mitochondrial generation of free radicals [196], thus, leading to an increase in cellular stress and eventually apoptosis. The extent to which Aβ receptor-linked pathways and internalized Aβ contribute to mitochondrial dysfunction and oxidative stress remains to be resolved.

1.4.4 Excitotoxicity

Glutamate, the main excitatory neurotransmitter in the CNS, binds to a variety of receptor-linked cation channels to modulate synaptic plasticity and learning and memory [197]. As noted before, excess amounts of glutamate lead to dysfunctional Ca^{2+} homeostasis [27, 198], and increase extracellular glutamate by inhibiting re-uptake [125, 126], subsequently eliciting excitotoxicity and cellular death. Glutamate receptor-mediated excitotoxicity has been linked to various diseases [199–201]. Additionally, Aβ has been implicated in triggering excitotoxicity in AD through sustained activation of NMDA (N-methyl-D-aspartate)-type and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamate receptors. This in turn, causes elevated intraneuronal Ca^{2+} levels due to excess glutamate from oxidative stress and Aβ accumulation [121, 122]. Of the two FDA-approved drugs on the market to treat AD, the NMDAR antagonist memantine effectively blocks prolonged receptor activity, improving patient behavior and cognition [130], making it a potential agent against glutamate-mediated neurotoxicity [95, 202, 203]. The success of memantine on improving AD behavioral symptoms coupled with the fact that other agents have also been shown to protect against excitotoxicity by inhibiting various glutamate receptor activities [92, 204–206] makes targeting glutamate toxicity an attractive pathway for neuroprotection.

1.4.5 Synaptic Dysfunction

Synapses are involved in information transfer in the CNS and synaptic dysfunction is a major factor in neurodegenerative diseases [207] causing severe cognitive decline [208]. In AD, the over-accumulation of Aβ continuously acts on NMDARs causing Ca^{2+} deregulation and oxidative stress [122] leading to synaptic loss and toxicity. Under these conditions, the activation of stress-kinases [209, 210] and caspases [211, 212] have been linked to synaptic and cognitive dysfunction. NMDA receptors have been extensively implicated in synaptic plasticity involved in learning and memory, and the abnormal stimulation of NDMA receptors leads to excitotoxicity (as mentioned earlier). In addition, reductions in NMDA receptors in the hippocampus and cortex of postmortem AD have also been observed [213]. Aside from utilizing NMDA receptor antagonists for neuroprotection against excitotoxicity, targeting downstream pathways may provide an alternative method to rescue synaptic toxicity by upregulating NMDA receptor expression and/or activity. Downstream targets of interest include CREB and its upstream regulator protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), calcineurin and protein phosphatase 1 (PP1) [214, 215]. BDNF, an important regulator in synaptic plasticity [148, 216], upregulates CREB, which in turn, enhances expression of BDNF in a feed-forward mechanism.

Autophosphorylation of CAMKII is associated with an increase in Ca^{2+}-independent protein kinase activity linked to LTP enhancement under physiological conditions, whereas dephosphorylation prevents LTP [217] and leads, eventually, to memory impairments. Specifically, activated CAMKII has been shown to translocate to

the nucleus in post-synaptic neurons upon Ca^{2+} influx through NMDA receptors [218] during LTP induction [219], mediating the downstream impact of NMDA receptor activation resulting in long-lasting molecular changes responsible for synaptic plasticity, learning and memory [220–222]. In postmortem tissue of AD patients, a significant loss of CAMKII-expressing neurons was observed in the hippocampus [223], and altered expression and activity of CAMKII were found in AD mouse models [224, 225]. Considering its evident loss of expression and activity in disease models, inhibitors of CAMKII-dephosphorylation (via phosphatase inhibition) may be another target to maintain synaptic integrity and function.

Calcineurin is another Ca^{2+}/calmodulin-dependent protein that is a protein phosphatase implicated in various signaling transductions. Dysfunction of calcineurin is also associated with pathological Ca^{2+} signaling in cognitive disorders and impairments in LTP and LTD. In vitro, excess Aβ increases the activity of calcineurin, which, in turn, decreases AMPA receptor function. This results in enhanced LTD [211] and decreased CAMKII activity [217] preventing LTP . Alternatively, inhibition of calcineurin reverses Aβ-dependent learning and memory impairments [226], reduces Aβ-induced neuronal and dendritic spine loss [227], and rescues early synaptic plasticity deficits [228]. Additionally, the pathophysiological activation of calcineurin activates the phosphatase PP1 [229], which negatively regulates PKA [230] and deactivates CAMKII [231], affecting synaptic plasticity and survival. Furthermore, calcineurin also activates the nuclear factor of activated T cells (NFAT) which subsequently leads to dendritic spine loss and neuritic dystrophies [232]. Thus, targeting PP1 activity may provide another neuroprotection avenue.

In the CNS, nitric oxide (NO) is a “gasotransmitter” (gaseous signaling molecule) that plays an important role in brain activity as a retrograde neurotransmitter or a mediator of toxicity [233]. In addition to being involved in vasodilation, NO is involved in learning and memory through LTP induction [234, 235] by interacting with soluble guanylyl cyclase (sGC) to increase cGMP (cyclic guanosine monophosphate) to elicit a cGMP-dependent release of glutamate [236], known as the NO-cGMP cascade. As previously mentioned, physiological levels of glutamate are necessary for synaptic plasticity, learning and memory [197], but under pathological conditions, the overproduction and accumulation of NO contributes to cell stress that leads to altered intracellular signaling, protein misfolding, mitochondrial damage, synaptic dysfunction and apoptosis [237, 238]. Restoring NO signaling in the brain to improve blood flow, modulate inflammation or enhance synaptic viability and function is already under investigation [239–241].

The mitogen-activated protein kinase (MAPK) cascades are a major signaling pathway for neuronal survival, proliferation and differentiation [242]. Of the key regulators in the MAPK pathway, activation of c-Jun amino-terminal kinase/stress-activated protein kinase (JNK) and p38 are strongly associated with LTP impairments [243, 244]. Studies have shown that inhibition of JNK and p38 improves synaptic plasticity and cognitive deficits [245, 246], suggesting that MAPK inhibitors may protect against synaptic dysfunction.

1.4.6 Apoptosis

Modulating neuronal survival signaling to inhibit apoptosis or upregulate anti-apoptotic pathways is another avenue for neuroprotection. Proteins in the Bcl-2 family, consisting of both pro-apoptotic and anti-apoptotic proteins, and members of the caspase

family play critical roles in controlling activation and transduction of apoptosis [247, 248]. The increase in pro-apoptotic activity results in mitochondrial dysfunction and release of cytochrome C [249] subsequently activating the intrinsic apoptotic pathway. In addition, increases in oxidative and ER stress activate the unfolded protein response (UPR) in the ER [250] causing ER stress-induced apoptosis. Compounds targeting apoptosis exert their neuroprotective effects by reducing pro-apoptotic Bcl-2 and caspase members [251–253] or by reducing ER-induced apoptosis [254, 255].

In addition to preventing apoptosis, modulating neuronal survival signals is another target for neuroprotection. Within the MAPK pathway, activity of the extracellular signal regulated protein (ERK), JNK and p38 are upregulated with elevated levels of Aβ *in vivo* [256] and *in vitro* [136], suggesting that the MAPK pathway is also associated with AD pathology. Inhibitors of ERK, JNK and p38 have been extensively studied as potential therapeutic agents against neurodegenerative diseases [257, 258], improving spatial learning and memory impairments and neuronal survival [259, 260].

1.4.7 Cell Survival

Neurotrophins play an important role in the development, maintenance, repair and survival of neurons. Studies have shown that neurotrophins inhibit cell death [261, 262] and promote neuronal survival [101] through the activation of multiple transcription factors, such as CREB, to induce the expression of prosurvival and prodifferentiation genes [263]. As previously noted, CREB is believed to upregulate neurotrophin expression and activity. Addittionally, CREB is also activated in response to stressful stimuli [264] through the MAPK [265], PI3K/AKT [266], NF-κB [267], and PLCγ/PKC and

cAMP/PKA [268] pathways leading to the expression of the pro-survival gene bcl-2 [147]. In light of the fact that decreased expression of BDNF plays an important role in various neurodegenerative disorders [269] and CREB has been shown to be downregulated in hippocampal neurons in AD models [105], a means to increase CREB activity may provide a potential neuroprotective avenue.

Another important survival pathway involved in synaptic plasticity, axonogenesis, neural development and neural homeostasis is the Wnt signaling pathway [270]. The activation of the Wnt pathway attenuates cytosolic glycogen synthases kinase 3β (GSK-3β), thereby increasing β-catenin nuclear translocation and subsequently upregulating proteins involved in neural development and homeostasis [270]. Activation of the Wnt pathway may provide an alternative target for neuroprotection [271, 272] with several groups already investigating molecules capable of upregulating this pathway [273, 274]. Taken together, the evidence shows that modulating the apoptotic or survival pathways may provide good targets for neuroprotection.

1.5 Concluding Remarks

Although a considerable amount of research and time has been spent exploring and developing protective therapies against Aβ toxicity, to date, none of these neuroprotective agents has yet been shown to provide neuroprotection in AD, as defined, namely altering or rescuing the course of the disease. The development of an effective neuroprotective compound against AD may be limited by a number of factors in addition to those already outlined. First, it is imperative to begin treatment with an agent able to penetrate the BBB, whether orally, systemically or intranasally administered, at the right

window of opportunity to ensure neuronal and synaptic protection against toxicity leading to any further damage. In some cases, the agent may also need to be able to penetrate intracellularly to act on intracellular targets. Second, low levels of Aβ have an important physiological function [36] and disruption of this homeostasis may contribute to toxicity. Third, neuroprotective therapeutics should not only preserve existing and surviving neurons but should also improve their function, including plasticity and adaptability. Lastly, neuroprotection against Aβ toxicity cannot be limited to a single target or pathway due to the fact that AD is a multifactorial disorder that involves multiple biological pathways (Fig. 3). Although the development of a potent and effective therapeutic against Aβ toxicity has yet to prove successful in clinical trials, progress is underway in identifying agents that will hopefully meet the standards of definition for neuroprotection in AD models in the near future.

There are currently only two classes of FDA-approved drugs for AD treatment, cholinesterase inhibitors and the NMDA-type glutamate receptor antagonist. Although these drugs are somewhat efficacious in treating the symptoms of AD for some patients over a limited period of time, unfortunately, none is capable of reversing the course of AD or even significantly slowing down the rate of progression. Therapeutic implementation of neuroprotective approaches offers, on the other hand, a direct means to prevent or reduce, if not reverse, disease progression. In a survey of the most recent agents with demonstrated protective activity toward Aβ toxicity, only a minor subset displays neuroprotection at the synaptic, cellular and behavioral levels [98, 275, 276].

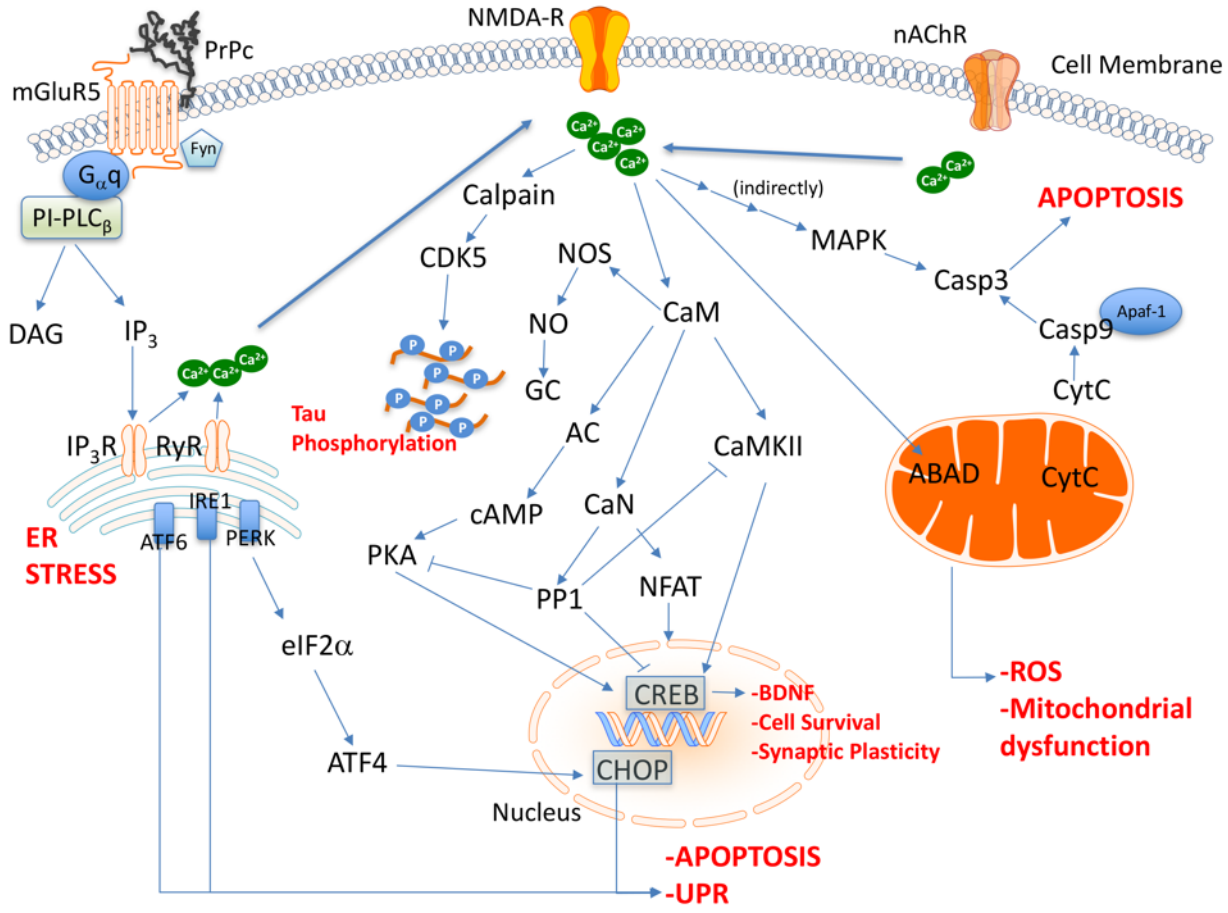


Figure 3: Prominent Aβ-induced Ca^{2+} Dysregulation Toxicity Pathway

Aβ-induced elevation of neuronal Ca^{2+} elicits various signaling cascades that subsequently induce apoptosis, mitochondrial dysfunction, oxidative stress, ER stress and synaptic dysfunction.

1.6 Hypothesis

The N-Aβcore, accounting for the neuromodulatory activity of Aβ, protects against Aβ-induced mitochondrial dysfunction, oxidative stress and apoptotic neuronal death, and rescues Aβ-induced synaptic dysfunction.

1.7 Specific Aims

1.7.1 Specific Aim 1

Aim 1A: To assess the N-Aβcore and its mutants for neuroprotection against Aβ-induced neurotoxicity.

Aim 1B: To characterize differential changes of presynaptic Ca^{2+} responses of the N-Aβcore and mutants.

Rationale: Preliminary data suggest that the main active part of Aβ and the N-Aβ fragment is the N-Aβcore [62], and Aβ monomers were found to be neuroprotective via the PI3-kinase/Akt pathway [128]. We propose that the core sequence is neuroprotective against Aβ-induced apoptosis.

1.7.2 Specific Aim 2

Aim 2A: To examine the neuroprotective effects of the N-Aβcore on Aβ-induced synaptic dysfunction

Aim 2B: To elucidate the neuroprotective pathway of the N-Aβcore.

Rationale: Previous work has shown that endogenous pM levels of Aβ and the N-Aβ fragment enhance synaptic plasticity [36, 62]. Additionally, the N-Aβ fragment prevents Aβ-induced inhibition of synaptic plasticity [62]. Therefore, we propose that the N-Aβcore can rescue Aβ-induced synaptic dysfunction. Furthermore, as an extensive amount of research has been focused on understanding Aβ toxicity and Aβ-independent

neuroprotective pathways, we aim therefore to elucidate the protective mechanism(s) of the N-Aβcore.

1.8 Significance

Characterization of the relative activity of N-Aβcore and mutants to protect against Aβ toxicity allowed optimization of a core sequence that will serve as a scaffold for synthesis of orally active peptidomimetics. Additionally, the understanding of the neuroprotective action of the N-Aβcore will provide additional insights to potential targets for AD therapeutics.

CHAPTER 2: THE NEUROPROTECTIVE EFFECTS OF THE N-AβCORE ON Aβ-INDUCED NEUROTOXICITY AND THE DIFFERENTIAL CALCIUM CHANGES OF THE N-AβCORE

2.1 Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disease characterized by synaptic and neuronal loss in regions of the brain critical for memory and cognition. Histologically, AD is characterized by tau containing neurofibrillary tangles and beta amyloid (Aβ) plaques [277], the latter resulting from the over-accumulation of soluble Aβ years prior to a diagnosis of AD (reviewed in [278]). In contrast, the levels of Aβ normally present in brain in the absence of AD, estimated to be in the picomolar (pM) range, have been found to enhance synaptic regulation [32, 33], synaptic plasticity [34–36] and fear memory [32]. In addition, Aβ is produced and released from the presynaptic terminal [279] in an activity-dependent manner [34] and has a remarkably high turnover rate [280]. Together, these findings suggest that Aβ functions physiologically as a neuromodulator. As Aβ accumulates to nanomolar (nM) levels or higher during the prodromal stages of AD, dysfunction of synaptic signaling and memory processing ensues [20, 124].

Aβ is produced through the sequential enzymatic cleavage of the amyloid precursor protein (APP) via β- and γ-secretases [58]. As Aβ is partially derived from the transmembrane (TM) domain of APP, the peptide has a C-terminal hydrophobic domain, which has been shown to largely but not exclusively account for its aggregation and neurotoxicity [59, 60]. In contrast, a larger hydrophilic domain resides on the N-terminal side of the peptide. It has been shown that a 15-16 amino acid N-terminal Aβ fragment

(N-Aβ fragment) can be produced from this hydrophilic domain via an α-secretase-linked pathway and is present at significant levels in cerebrospinal fluid (CSF) [15].

Previous work indicated that the Aβ N-terminal domain is non-toxic [281]. Through structure-function analysis, we have shown that Aβ's neuromodulatory agonist-like activity toward nicotinic acetylcholine receptors (nAChRs) is contained within the hydrophilic N-terminal domain [62]. Moreover, we discovered that at pM-nM concentrations, the N-Aβ fragment is approximately twice as effective as Aβ in stimulating receptor-linked increases in Ca^{2+}, enhancing long-term potentiation (LTP) and enhancing contextual fear conditioning (CFC) [62]. Additionally, treatment of hippocampal slices from transgenic mice expressing the Swedish mutation APP (hAPPswe: B6.SJL-Tg(APPswe:APP_{695}K670N,M671L)2576Kha) with the N-Aβ fragment rescued LTP deficits shown to exist in this APP-AD mouse model [62] as the result of elevated Aβ. We further narrowed down the core sequence accounting for the activity of the N-Aβ fragment, identifying an essential and unique core hexapeptide sequence, YEVHHQ (N-Aβcore). It was found to be as effective as the N-Aβ fragment in eliciting Ca^{2+} responses through nAChRs [62].

To investigate the possible impact of the N-Aβcore on full-length Aβ-induced neuronal toxicity in a model neuronal system (neuroblastoma cell line NG108-15), we studied Aβ-triggered Ca^{2+} responses, oxidative stress, and apoptosis offers a novel avenue for the development of AD therapeutics.

2.2 Methods

2.2.1 Neuroblastoma Clonal Cell Culture and Transfection

Rodent hybrid neuroblastoma NG108-15 cells (courtesy of Dr. William Atchison, Michigan State University) were used as a model nerve cell system allowing reconstitution with defined target receptors for Aβ [146]. The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) and differentiated in the presence of reduced serum (1% FBS) and 1mM dibutyryl cyclic AMP for 72h. Under these differentiation conditions, the cells extend axonal processes having presynaptic varicosities (identified morphologically and by presynaptic markers) capable of forming cholinergic synapses with the appropriate postsynaptic target [282]. Expression vectors (pcDNA3.1) containing mouse sequences for α4 and β2 nAChR subunits (courtesy of Dr. Jerry Stitzel, University of Colorado) were transiently transfected into the cells at 1:4 ratio, respectively, using FuGENE HD (ThermoFisher, catalog # PRE2311) for 48h. Mock-transfected NG108-15 cells, exposed only to FuGENE HD and not plasmid DNA, were used as controls [33].

2.2.2 Confocal Imaging of Intracellular Calcium

Changes in intracellular Ca^{2+} levels in individual varicosities of differentiated NG108-15 cells were monitored by the Ca^{2+}-selective fluorescent dye Fluo-4, as described [146]. Fluo-4/AM (Invitrogen, catalog # F23917) was loaded into the differentiated cells cultured on Cell-Tak-coated coverslips. Fluo-4-loaded cultures were perfused with oxygenated HEPES-buffered saline (HBS: 142 mM NaCl, 2.4 mM KCl, 1.2 mM K_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM D-glucose, 10 mM HEPES (pH 7.4), 100

nM tetrodotoxin, TTX) via a rapid exchange Warner perfusion chamber. Changes in fluorescent intensity (F) in response to various treatments (Aβ alone or in combination with N-Aβ fragment or N-Aβcore with pretreatment, co-treatment or post-treatment; Aβ or the N-Aβ fragment followed by co-treatment with the N-Aβcore) were visualized by a Nikon PCM 2000 Chameleon confocal imaging system. Each time-series was normalized to baseline fluorescence intensity at time zero (F_0) to yield the relative change in $Ca^{2+}i$ (as F/F_0). Peak responses for four to ten varicosities per sample were collected during 60–180s after the initiation of stimulation and determined across all frames using ImageJ software.

2.2.3 Mitochondrial Membrane Potential

The mitochondrial membrane potential was measured using TMRE (tetramethyl rhodamine ethyl ester)- mitochondrial membrane potential assay kit (Invitrogen, catalog # T669). Briefly, nAChR-transfected NG108-15 cells were treated over one to five days with either Aβ or Aβcore. The cells were incubated with 50nM TMRE in HBS for 20 minutes and imaged live using an Olympus IX71 epifluorescence microscope at excitation/emission of 549/575nm, respectively.

2.2.4 Reactive Oxygen Species (ROS)/ Hoechst staining

Oxidative stress was determined by changes in the levels of ROS using the Image iT Live Reactive Oxygen Species (ROS) Detection kit (Invitrogen, catalog # I36007) [283]. In brief, NG108-15 cultures were subjected to various treatments for three days. The medium for each treatment condition was changed every day, unless otherwise noted. At

the end of the treatment periods, the cells were incubated with carboxy-H_2DCFDA (component A) at 37°C for 30 min. During the last 5 min of incubation, 2 μg/ml of HOECHST stain (component B) was added to assess, in parallel, the integrity of the cell nuclei. The cells were washed twice with HBS and visualized using an Olympus IX71 or IX81 epifluorescence microscope at excitation/emission of 495/529 nm (ROS) and 350/461 nm (HOECHST), respectively.

2.2.5 Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) Assay

Apoptosis was measured using TUNEL staining via a Click-iT TUNEL Alexa Fluor488 Imaging Kit (Invitrogen, catalog # C10245) in accordance with the manufacturer's protocol [136]. In brief, the cells were subjected to various treatments for four days, exchanging the medium each day. After the fourth day of treatment, the cells were fixed with freshly prepared 4% paraformaldehyde (neutralized with NaOH while heating to produce formaldehyde) in PBS for 20 min and permeabilized with Triton X-100 (0.25% in PBS) for another 20 min. The cultures were then washed twice and incubated with 50μL of terminal deoxynucleotidyl transferase reaction buffer (Component A) for 10 min. The buffer was replaced with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and incubated in a humidified chamber at 37°C for 60 min. The cells were then washed three times with 3% bovine serum albumin (BSA) in PBS for 2min each and thereafter, incubated with 50μL of Click-iT reaction mixture (containing Alexa 488 azide) for 30 min at room temperature, protected from light exposure. The cells were again washed with 3% BSA in PBS and the cell nuclei were counterstained with

Hoechst 33342 for 15 min at room temperature, protected from light. The coverslips were washed twice with PBS before mounting onto a slide with Vectashield mounting medium (Vector Laboratories, Burlingame,CA).

2.2.6 Human Aβ and Derivatives

Full-length Aβ and the N-Aβ fragment were obtained as hydrochloride salts from American Peptide/ BACHEM (Aβ: catalog # 62-0-80; N-Aβ fragment: 62-1-04) or Anaspec (Aβ: catalog # AS-21793; N-Aβ fragment: AS-61798). The N-Aβcore and mutants were custom-ordered from Peptide 2.0. (Comparison of the peptides from various sources yields equivalent neuromodulatory activities. There is no evidence that the N-Aβcore has an effect on Aβ oligomer formation.) All peptides were synthesized and isolated at >98% purity, as assessed by mass spectrometry and high-performance liquid chromatography. Aβ (1-42), the N-Aβ fragment (1-15), and the N-Aβcore (10-15) and mutant forms were solublized in double-distilled water and used at pM to nM final concentration in buffered saline [33].

2.2.7 Reagents

All standard reagents (buffers, salts, tissue culture media and substrates, paraformaldehyde, Triton X-100, etc.) were obtained from Sigma or ThermoFisher and were of the highest grade available (>98% purity). Plasmid purification maxiprep kits were from Qiagen (catalog # 12262) or Zymo Research (catalog# D4202). FBS was purchased from Omega Scientific (catalog # FB-11, Lot # 514205). Tetrodotoxin was purchased from Abcam (catalog # ab120054, Lot # GR169765). BD Cell-Tak was obtained from VWR (catalog # 47743-684, Lot # 7100009).

2.2.8 Statistical Analyses

Treatment and units were randomized as to order for all assays and experiments. Biological replicates were based on independent samples (*n*). All experiments were repeated at least three times. All quantitative results are presented as boxplots (5-95% confidence intervals), where appropriate, or means ± SEM. Statistical analyses were performed using Prism (GraphPad v5.0b; RRID:SCR_002798). Multiple comparisons were made using one-way ANOVA with Bonferroni or Tukey post hoc tests, as indicated. Paired comparison was made using Student's *t*-tests. *P*-values <0.05 were considered the minimum for significance (as rejection of the null hypothesis).

2.3 Results

2.3.1 N-Aβcore and N-Aβ fragment are highly effective, potent activators of α7- and α4β2-nAChRs

We have previously shown that the N-Aβcore (10-15) and N-Aβ fragment (1-15) have similar potencies to that of $A\beta_{1-42}$ as neuromodulators, measured as changes in presynaptic Ca^{2+} in the axonal varicosities of α7-nAChR-transfected, differentiated neuroblastoma NG108-15 cells used as a reconstituted model neuronal system [62]. In order to determine the key functional residues in the core sequence, YEVHHQ, screening of numerous N-Aβcore mutants was performed using Fluo-4 to measure relative changes in intracellular Ca^{2+}. We found that mutating the His-13 and His-14 to Ala-13 and Ala-14 (H13A,H14A), respectively, reduced activity compared to the wild-type N-Aβcore

(p<0.001), whereas mutating the Gln-15 to His-15 (Q15H) showed a trend towards an increase in activity (Fig. 4A).

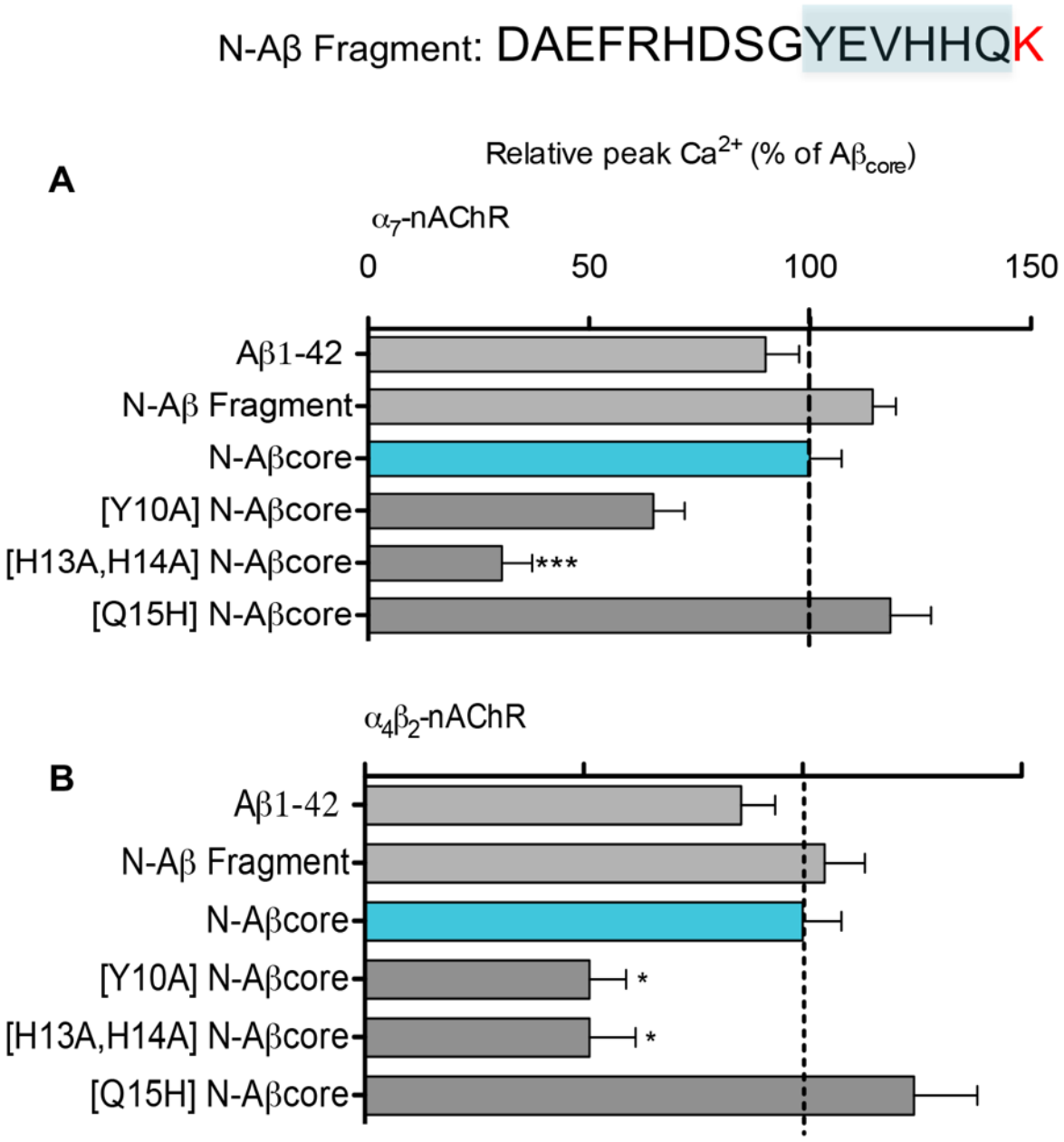


Figure 4: Mutational-analysis of the N-Aβcore using NG108-15 cells expressing α7- or α4β2-nAChRs.

Top, the human N-Aβ fragment sequence, highlighting the N-Aβcore (shaded) A, Relative average peak Ca^{2+} responses in the axonal varicosities of differentiated NG108-15 cells expressing α7-nAChRs to 100nM $Aβ_{1-42}$ (*n*=44), N-Aβcore (*n*=70), N-Aβ fragment (*n*=178), [Y10A] N-Aβcore (*n*=14), [H13A,H14A] N-Aβcore (*n*=19), and [Q15H] N-Aβcore (*n*=33). B, Relative average peak Ca^{2+} responses in varicosities of differentiated NG108-15 cells expressing α4β2-nAChRs to 100nM $Aβ_{1-42}$ (*n*=36), N-Aβcore (*n*=32), N-Aβ fragment (*n*=35), [Y10A] N-Aβcore (*n*=21), [H13A,H14A] N-Aβcore (*n*=19), and [Q15H] N-Aβcore (*n*=29). *n* refers to the number of individual varicosities. Averaged data are means ± SEM, normalized to the average peak Ca^{2+} responses N-Aβcore (dashed lines). *p<0.05 and ***p<0.0001 (Bonferroni *post hoc* tests for comparison with the N-Aβcore).

An alanine scan across the remaining residues of the N-terminal of the N-Aβcore resulted in no significant change in the α7-nAChR-linked Ca^{2+} responses (Table 1). These findings suggest that His-13 and His-14 are critical in eliciting activity, which is consistent with previous mutational analysis of N-Aβ fragments [284, 285] as well as full-length Aβ [62, 286].

Table 1. Averaged Ca^{2+} changes in NG108-15 cells on stimulation with N-Aβcore alanine and truncation mutants.

		Changes in $Ca^{2+}i$	
		Mean ± SD	n
α_7	[Y10S] N-Aβcore	38.72 ± 0.18	10
	[E11A] N-Aβcore	73.10 ± 0.30	25
	[V12A] N-Aβcore	82.42 ± 0.24	21
	[11-14] N-Aβcore	68.97 ± 0.25	11
	[12-15] N-Aβcore	73.00 ± 0.82	18
$\alpha_4\beta_2$	[Y10S] N-Aβcore	42.12 ± 0.43	13
	[E11A] N-Aβcore	70.70 ± 0.64	34
	[V12A] N-Aβcore	71.40 ± 0.25	25

Average peak Ca^{2+} responses in axonal varicosities of differentiated NG108-15 cells expressing α7-nAChRs or α4β2-nAChRs to various N-Aβcore alanine and truncation mutants, expressed as % ± S.D. of the average peak Ca^{2+} response to the N-Aβcore. N is the number of varicosities.

To translate these findings into our neuronal toxicity model, we performed a similar set of experiments with α4β2-nAChR-transfected cells, which were previously shown to respond to $Aβ_{1-42}$ in an α4-dependent manner [136]. Using the same approach, we demonstrated that the N-Aβcore and the N-Aβ fragment retain their neuromodulatory

activity at nM (Fig. 4B; Fig. 5) and pM (Fig. 5) concentrations. In accordance with our earlier findings, the H13A,H14A mutation reduced activity compared to the N-Aβcore (p<0.05), whereas the Q15H mutation showed a trend towards an increase in activity (Fig. 4B). In contrast, mutation of Glu-11 and Val-12 to Alanine showed no difference in activity (Table 1). However, a significant reduction was observed on mutating Tyr-10 to an Alanine when compared to the wild-type N-Aβcore (Fig. 4B; p<0.05 vs. wild-type). Mutation of Tyr-10 to a Serine also significantly reduced activity in both α7- and α4β2-nAChR-transfected cells (Table 1), with a trend evident in the truncation mutants with removal of this tyrosine. This loss in activity suggests that the tyrosine residue also plays a role in the N-Aβcore's activity toward α4β2-nAChRs, possibly through the aromatic and hydroxyl moieties [33]. We have previously shown that Tyr-188 in the ligand binding domain of α7 nAChR plays an essential role in the agonist action of Aβ in presynaptic regulation [33] and thus ligand-target aromatic side chain interactions may be involved.

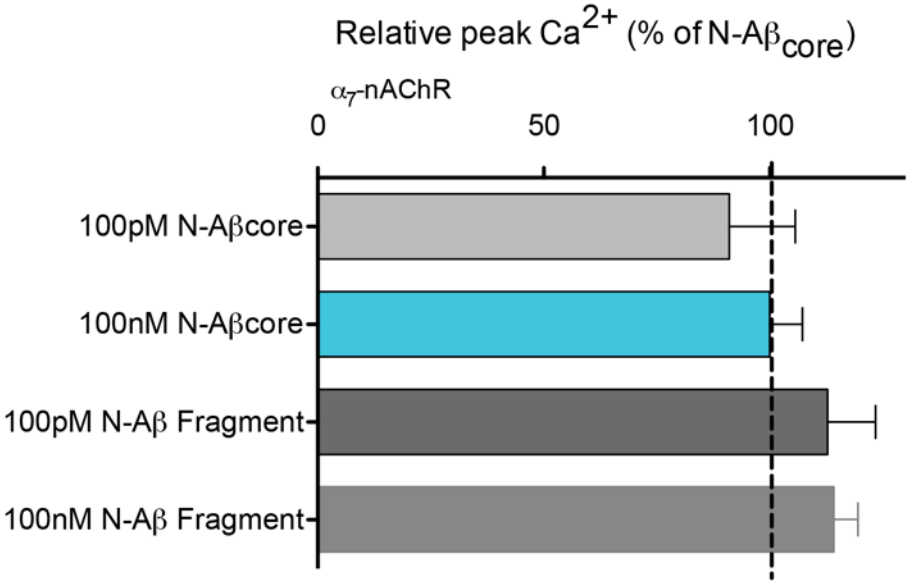


Figure 5: Lower concentrations of N-Aβcore and N-Aβ fragment retained significant Ca^{2+} activity.

Average peak Ca^{2+} responses in axonal varicosities of NG108-15 cells expressing α7-nAChRs to 100pM N-Aβcore (*n*=8), 100nM N-Aβcore (*n*=70), 100pM N-Aβ fragment (*n*=32) or 100nM N-Aβ fragment (*n*=178). Averaged data are means ± SEM and dashed lines indicate the average maximal responses of N-Aβcore. *n* is the number of varicosities.

2.3.2 Prolonged treatment with the N-Aβcore elicits a transient Ca^{2+} response, whereas the N-Aβ fragment induces a sustained but attenuated response compared to full-length Aβ

To mimic prolonged exposure of neurons to amyloid peptides, we measured the effects of $Aβ_{1-42}$, N-Aβcore or N-Aβ fragment on α4β2-nAChR-linked Ca^{2+} responses in our neuronal model over an extended period of time. Treatment with 100nM $Aβ_{1-42}$ induced a long, sustained increase in Ca^{2+} out to tens of minutes (Fig. 6A), similar to previous findings [146]. The N-Aβ fragment (100nM) also elicited a sustained but significantly reduced response. In contrast, 100nM N-Aβcore induced only a transient spike in activity that peaked at 2 min of treatment, returning back to baseline after 4 min (Fig. 6A). These findings suggest that the N-Aβcore and N-Aβ fragment differentially couple to target receptors to induce changes in internal Ca^{2+} in comparison to the sustained changes on prolonged exposure to $Aβ_{1-42}$, which likely lead to dysregulation of mitochondria [136, 287]. The extent to which these differences in primary signaling account for the absence of toxicity for these N-Aβ fragments remains to be determined. However, co-treatment with N-Aβcore following prolonged application of $Aβ_{1-42}$ or the N-Aβ fragment strongly attenuated the Ca^{2+} responses (Fig. 6B).

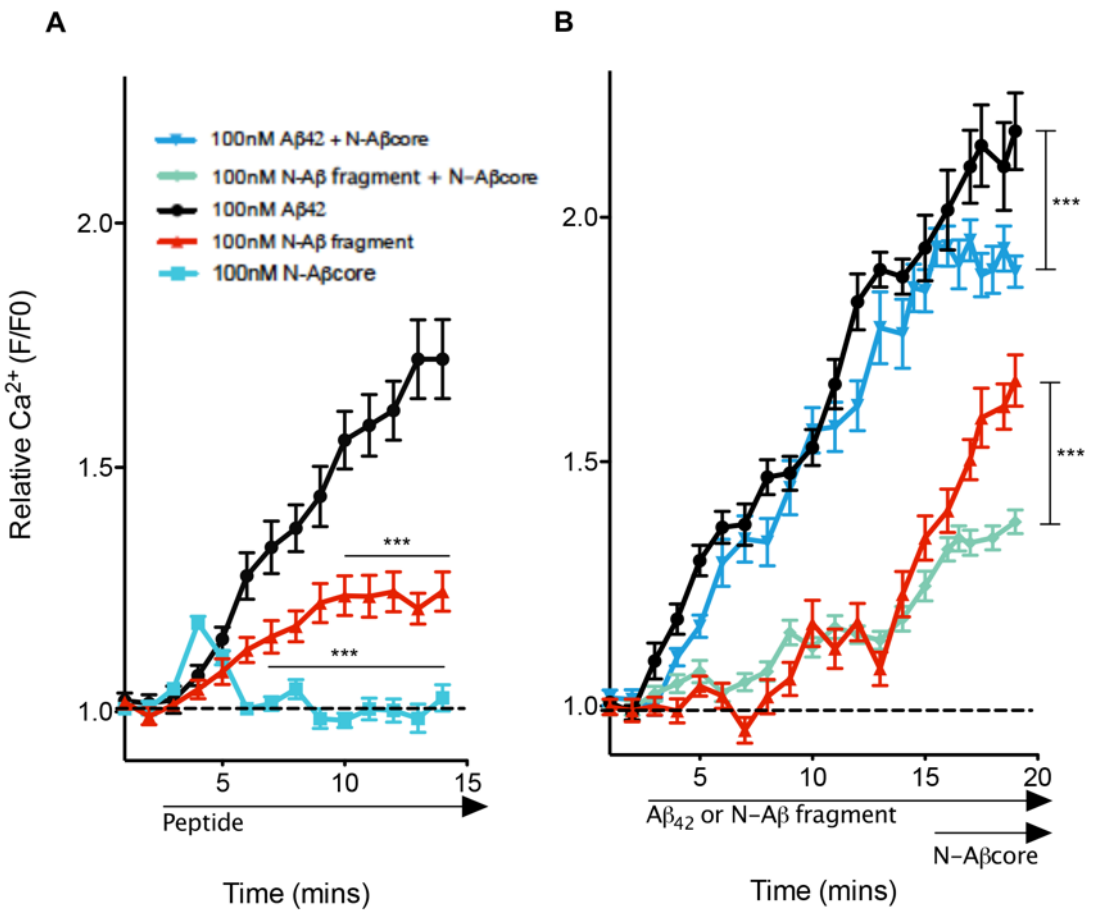


Figure 6: The N-Aβcore elicited a short-lived Ca^{2+} response compared to Aβ.

Average normalized Ca^{2+} responses (F/F_0) in the axonal varicosities of differentiated NG108-15 cells expressing α4β2-nAChRs to (A) 100nM N-Aβcore (*n*=33), 100nM N-Aβ fragment (*n*=17) or 100nM $Aβ_{1-42}$ (*n*=40) over 13 mins or (B) 100nM $Aβ_{1-42}$ (*n*=12) or 100nM N-Aβ fragment (*n*=10) for 20 mins (control) or for 15 mins followed by co-administration of 100nM N-Aβcore for 5 additional mins (*n*=32). Averaged data are means ± SEM, F_0 = fluorescence at time 0, and *n* represents the total number of varicosities examined across experiments. ***p<0.001 (Bonferroni *post hoc* tests of averaged plateau values).

2.3.3 The N-Aβcore rescues mitochondrial dysregulation induced by prolonged $Aβ_{1-42}$ exposure

Mitochondrial damage and dysfunction in AD due to the dysregulation of Ca^{2+} signaling has been widely reported [288, 289]. We have previously shown that prolonged exposure to $Aβ_{1-42}$ in our toxicity model altered mitochondrial dynamics, transport and

size [136]. Here, we examined the integrity of the mitochondrial membrane potential over five days of $A\beta_{1-42}$ treatment using the cell-permeant mitochondrial membrane potential probe TMRE. Depolarization of the membrane potential, indicated by a decrease in TMRE fluorescence across the cell population, was evident after just one day of $A\beta_{1-42}$ treatment (Fig. 7A), consistent with previous findings indicating that mitochondrial dysfunction is one of the earliest events in Aβ neurotoxicity [136]. Treatment for two days and longer showed a greater extent of inactivation of the membrane potential, indistinguishable from 2 days on (Fig. 7A). Co-treatment with the N-Aβcore (3-day timeframe) prevented the $A\beta_{1-42}$-triggered depolarization of the mitochondrial membrane potential (Fig.7B).

2.3.4 The N-Aβcore and the N-Aβ fragment protect against full-length Aβ-induced oxidative stress

We have previously shown that prolonged treatment with $A\beta_{1-42}$ induces oxidative stress (ROS), nuclear disintegration and apoptotic cell death in our *in vitro* neuronal toxicity model [136]. To assess the potential neuroprotective effects of the non-toxic N-Aβcore and N-Aβcore mutants against $A\beta_{1-42}$-induced toxicity, we assessed these indicators following administration of the N-terminal peptides under various conditions with daily treatment with 100nM $A\beta_{1-42}$ (Fig. 9). As previously shown, the presence of α4β2-nAChRs sensitizes the neuroblastoma NG108-15 cells to the toxicity of $A\beta_{1-42}$ at nM concentration. In contrast, co-treatment of α7-nAChR-transfected cells with the N-Aβ fragment and $A\beta_{1-42}$ did not significantly protect against elevated ROS levels (Fig. 8). Co-treatment with the N-Aβcore at similar or higher concentrations was able to prevent $A\beta_{1-42}$-induced oxidative stress (Fig. 9B). The rescue treatment, in which the N-Aβcore was

introduced into the culture for 3-, 2-, or 1-day reduced $A\beta_{1-42}$-induced ROS back to baseline levels (Fig. 9B), suggesting that the N-Aβcore also protects against of $A\beta_{1-42}$-induced toxicity, including late stages of oxidative stress.

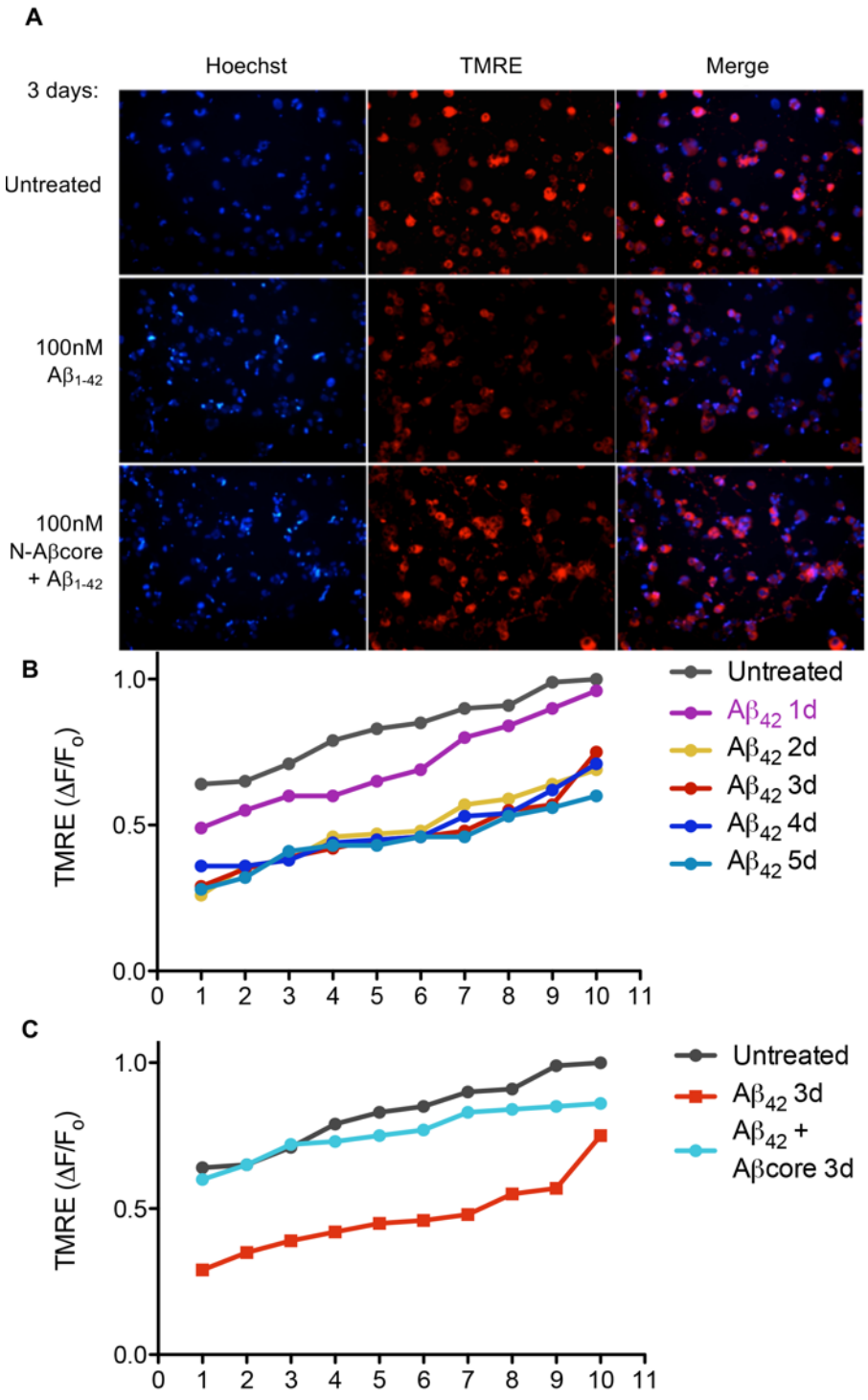


Figure 7: The N-Aβcore protected against Aβ-induced mitochondrial membrane dysregulation.

A, Differentiated NG108-15 cells stained with TMRE (50nM) or Hoeschst (nuclei) following 3 days of no treatment (Untreated control), 100nM $A\beta_{1-42}$ treatment or 100nM $A\beta_{1-42}$ treatment plus 100nM N-Aβcore. B, Integrated values ($\Delta F/F_0$) for TMRE associated with individual cells (randomly numbered) treated for 1-5 days with 100nM $A\beta_{1-42}$ (top graph) or 3 days with 100nM $A\beta_{1-42}$ plus 100nM N-Aβcore (bottom graph) are plotted as ranked distributions.

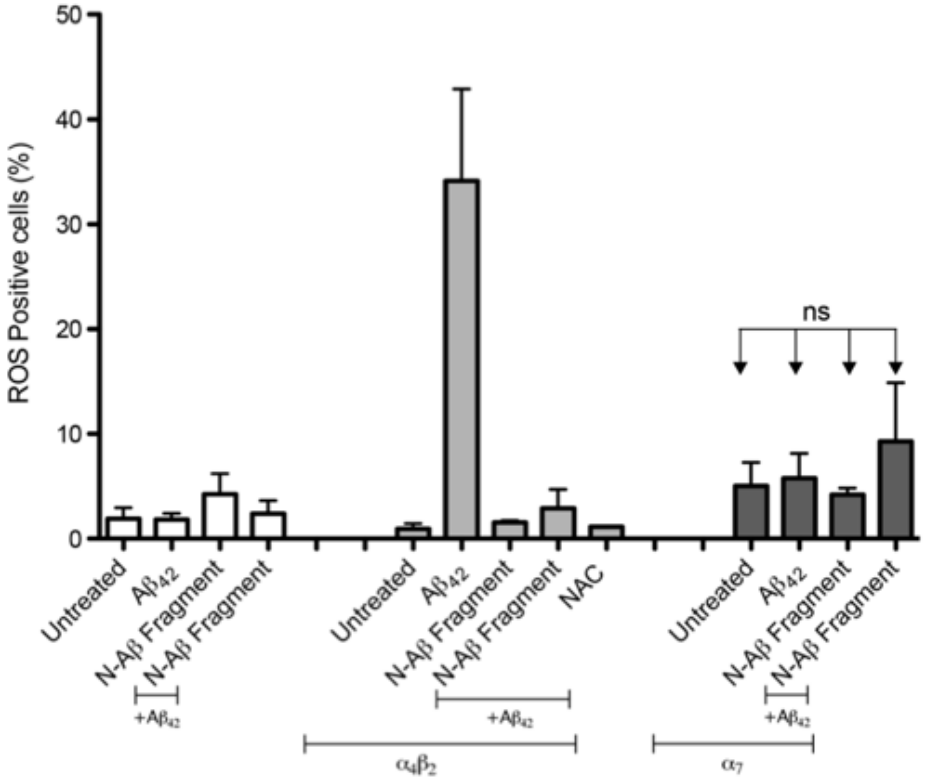


Figure 8: Treatment of α7-nAChR-transfected cells with $A\beta_{1-42}$ did not induce elevated levels of ROS.

Daily treatment with either 100nM $A\beta_{1-42}$ or the N-Aβ fragment alone or together in α7-nAChR-transfected NG108-15 cells (*n*=3). Mock-transfected cells are represented by open bars; nAChR-transfected cells are represented by closed (gray) bars. Quantification is percent of mean cell counts per experimental *n* (total number of cells). Averaged data are means ± SEM.

In mock-transfected cells (absent nAChRs), higher concentrations (μM) of $A\beta_{1-42}$ are required to induce significant levels of ROS. Co-treatment with nM-μM concentrations

N-Aβcore or N-Aβ fragment reduced this oxidative stress compared to $A\beta_{1-42}$ alone (Fig. 9C), demonstrating that the N-Aβcore or N-Aβ fragment can protect against $A\beta_{1-42}$–induced toxicity independently of $\alpha 4\beta 2$-nAChRs. Other studies have demonstrated that Aβ, at higher concentrations, was able to exert its effects independently of identified receptors in neuronal cells [287].

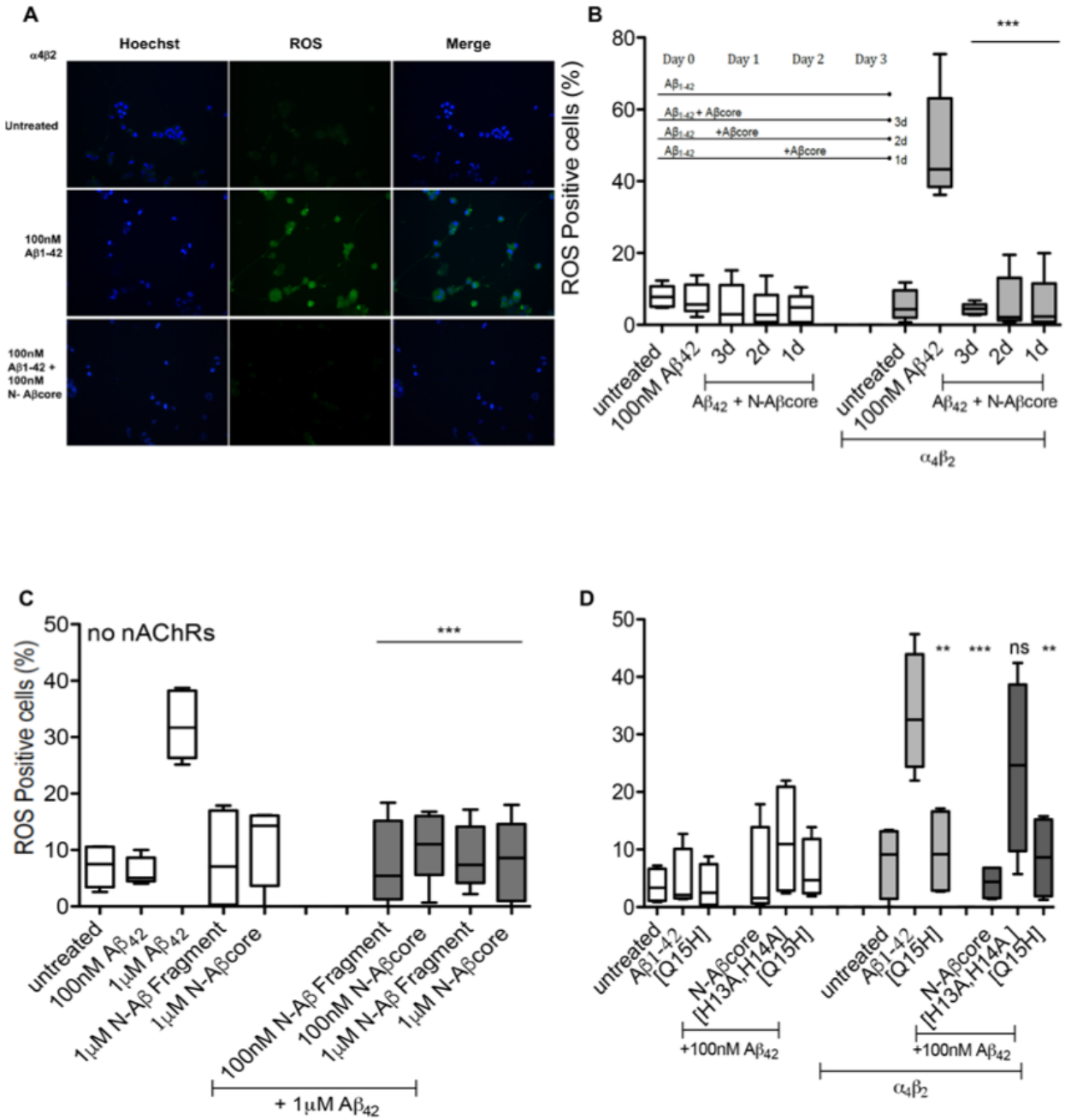


Figure 9: The N-Aβcore and the N-Aβ fragment protected against Aβ-induced oxidative stress in differentiated NG108-15 cultures.
A, Representative images of Hoechst and ROS staining. B, Treatment with 100nM N-Aβcore after 0-, 1-, 2-day treatment of 100nM $A\beta_{1-42}$ in the presence α4β2-nAChR (*n*=5). C, Treatment of 100nm or 1μM N-Aβcore or N-Aβ fragment alone and in combination with 1μM $A\beta_{1-42}$ (*n*=5). Note that in the absence of nAChRs, μM concentrations of $A\beta_{1-42}$ are required to increase ROS. Co-treatments with $A\beta_{1-42}$ are represented by closed (grey) bars. D, Co-treatment of 100nM $A\beta_{1-42}$ with 100nM [H13A,H13A] N-Aβcore or 100nM [Q15H] N-Aβcore (*n*=4). For all experiments, mock-transfected cells are represented by open bars; α4β2-nAChR-transfected cells are represented by closed (gray) bars. Quantification is ROS-positive cells as a percentage of mean cell counts per experimental *n* (total number of cells). Data are represented via box-and-whisker plots across 5-95 percentile range, with the lines indicating the median values. *p<0.05, **p<0.001, ***p<0.0001 (Tukey *post hoc* tests compared to positive $A\beta_{1-42}$ control). ns, not significant.

We further examined whether the inactive [H13A,H14A] and highly active [Q15H] N-Aβcore mutants had any protective effect on elevated oxidative stress from $A\beta_{1-42}$. We found that co-treatment with nM concentrations of the active mutant reduced ROS compared to $A\beta_{1-42}$ alone (p<0.001), whereas the inactive mutant had no significant effect (Fig. 9D) at the concentration tested. These results substantiate the specificity of action of the N-Aβcore in neuroprotection against $A\beta_{1-42}$-induced oxidative stress.

2.3.5 Prolonged exposure to N-Aβcore or N-Aβ fragment protects against $A\beta_{1-42}$-induced apoptosis

In addition to inducing oxidative stress, we have previously shown that the presence of α4β2-nAChRs sensitizes differentiated neuroblastoma NG108-15 cells to $A\beta_{1-42}$-induced apoptosis [136], while elevated $A\beta_{1-42}$ triggers apoptosis in the absence of nAChRs. Therefore, we assessed whether the N-Aβcore or the N-Aβ fragment can protect against $A\beta_{1-42}$-induced apoptosis with or without the presence of α4β2-nAChRs. Co-treatment with nM concentrations of the N-Aβcore in the presence α4β2-nAChRs on

day 1, 2 or 3 following the start of daily $A\beta_{1-42}$ treatment reduced apoptosis compared to $A\beta_{1-42}$ alone (p<0.0001), as measured by TUNEL staining for apoptotic cells (Fig. 10A), suggesting that the N-Aβcore can protect against late stages of $A\beta_{1-42}$-induced neuronal death. In addition, high concentrations of $A\beta_{1-42}$ induced apoptosis in the absence of the sensitizing α4β2-nAChRs, and co-treatments with nM-μM concentrations N-Aβcore or N-Aβ fragment over four days also reduced DNA-fragmentation compared to $A\beta_{1-42}$ alone (p<0.0001 for 100nM and 1μM N-Aβcore; p<0.001 and p<0.0001, 100nM and 1μM N-Aβ fragment, respectively), with a trend of greater reduction at μM concentrations (Fig. 10B). These results indicate that the N-Aβcore or N-Aβ fragment can also protect against $A\beta_{1-}$

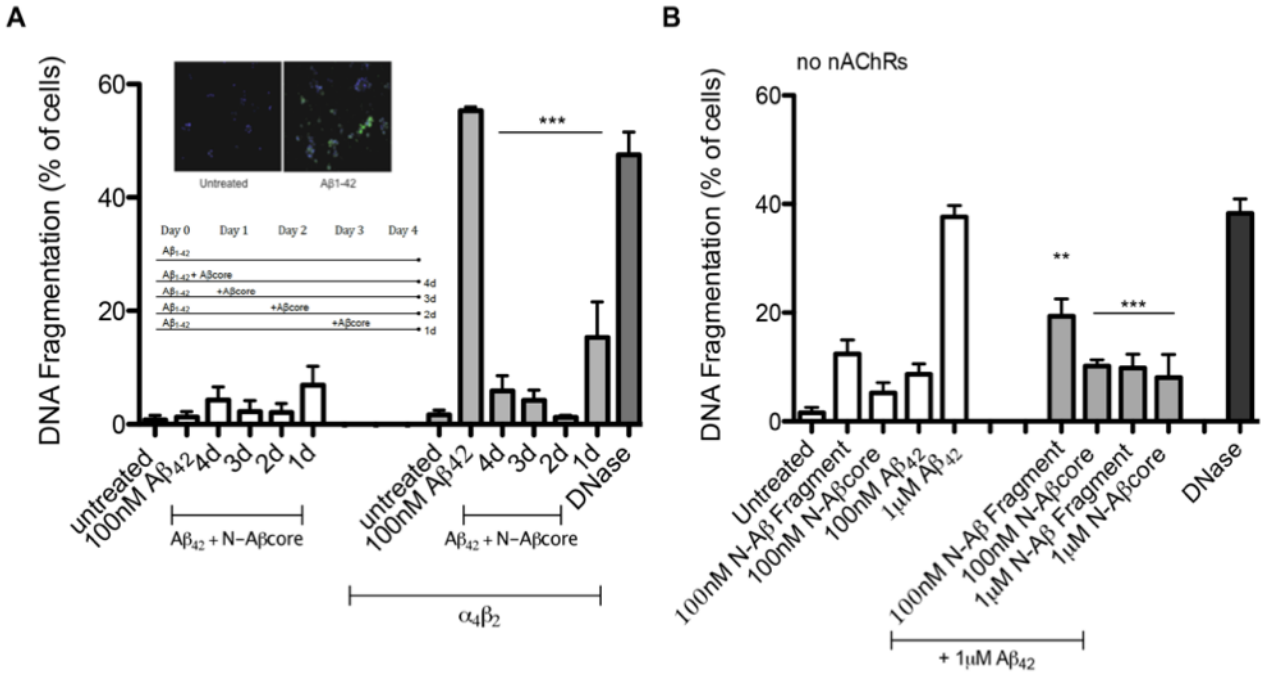


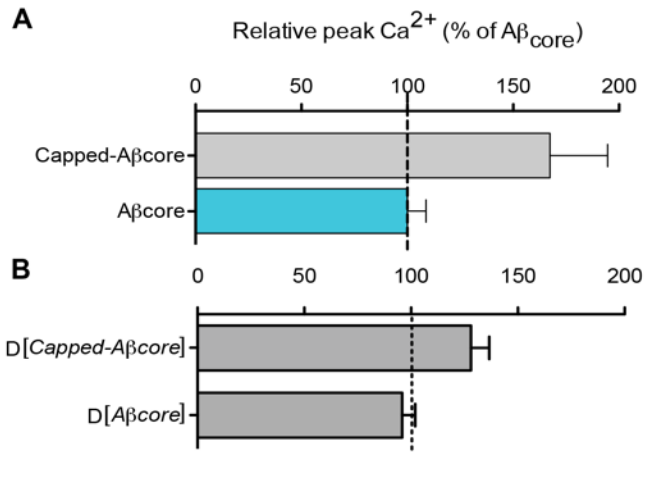
Figure 10: The N-Aβcore protected against $A\beta_{1-42}$-induced apoptosis.

A, Co-treatment with 100nM N-Aβcore after 0-, 1-, 2-, 3-day treatment of 100nM $A\beta_{1-42}$ in the presence of α4β2-nAChRs (n=3). Mock-transfected cells are represented by open bars; α4β2-nAChR-transfected cells are represented by closed (gray) bars. B, Daily treatment of 100nM or 1μM N-Aβcore or N-Aβ fragment alone and in combination with 1μM $A\beta_{1-42}$ (n=3). Note that in the absence of nAChRs, μM concentrations of $A\beta_{1-42}$ are required to induce cell death. Co-treatments with Aβ are represented by closed (gray) bars. Quantification is the percent of mean DNA fragmentation per experimental n determined from TUNEL staining. n is the number of cells. Averaged data are means ± SEM, **p<0.001, ***p<0.0001 (Tukey *post hoc* tests compared to positive $A\beta_{1-42}$ control).

$_{42}$-induced neuronal death independently of the α4β2-nAChRs.

2.3.6 Stabilization of N-Aβcore retains its receptor-linked Ca^{2+} activity and protects against $Aβ_{1-42}$-induced neurotoxicity in α4β2-nAChR-transfected cells

To protect the N-Aβcore from exopeptidase degradation *in vivo*, we capped its N-terminus with an acetyl group and its C-terminus with an amide group. Relative to the N-Aβcore, the capped-N-Aβcore retained its potent activity (Fig. 11A), with a trend towards an increased Ca^{2+} response. To further protect the N-Aβcore from endopeptidase activity, the enantiomeric conversion from the L-configuration to the D-configuration for each amino acid retained activity (Fig. 11B). In addition, the stabilized N-Aβcore, at nM concentrations, was as neuroprotective against $Aβ_{1-42}$-induced oxidative stress as the N-Aβcore (Fig. 11C; $p<0.05$ vs. $Aβ_{1-42}$ alone), indicating the potential for use of the stabilized peptide *in vivo*.



Aβcore	L-Core Sequence (10-15)
Capped- Aβcore	L-Acetyl-10-15-C-Amide
D[*Aβcore*]	D-Core Sequence (10-15)
D[*Capped- Aβcore*]	D-Acetyl-10-15-C-Amide

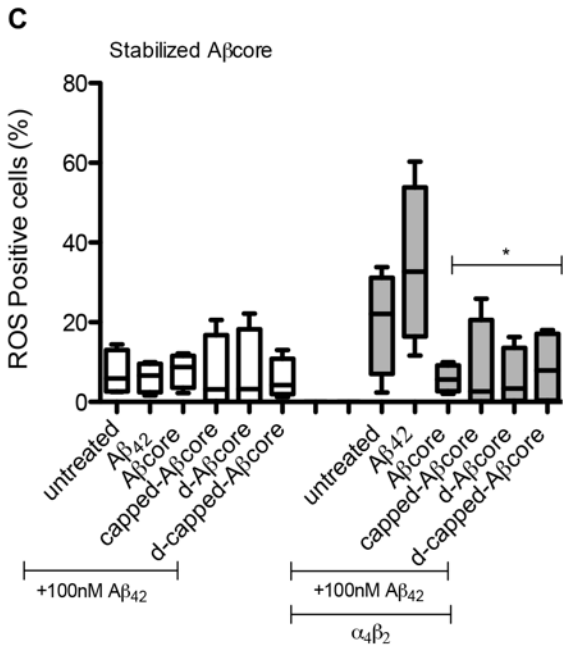


Figure 11: Stabilization of the N-Aβcore retained activity and protected against $A\beta_{1-42}$ - induced oxidative stress.

A, Average peak Ca^{2+} responses in varicosities of cells expressing $\alpha4\beta2$-nAChRs to 100nM N-Aβcore (*n*=51) and Capped-Aβcore (*n*=23). Protection of N-Aβcore via N-terminal acetylation and C-terminal amidation ("Capped") retains activity compared to N-Aβcore. B, Peak responses to 100nM *D*-[Aβcore] (*n*=57) and Capped-D-[Aβcore] (*n*=65). Enantiomer conversion and stabilization of N-Aβcore retains activity. Averaged data are mean ± S.E.M, and dashed lines indicate the average maximal responses of N-Aβcore. C, Daily co-treatment with 100nM $A\beta_{1-42}$ and 100nM capped-Aβcore, D-[Aβcore] or Capped-D-[Aβcore] reduces ROS. Mock-transfected cells are represented by open bars; $\alpha4\beta2$-nAChR-transfected cells are represented by closed (gray) bars. Quantification is percent of mean cell counts per experimental *n*. *n* is the number of varicosities. Percent positive ROS cells represented by 5-95 percentile box-and-whiskers plots, *p<0.05 (Bonferroni *post hoc* tests compared to positive $A\beta_{1-42}$ control).

2.4 Discussion

A broad range of studies have shown that synaptic and behavioral dysregulation resulting from Aβ is mainly due to soluble oligomeric forms of the peptide, while neuronal toxicity leading to neurodegeneration may be triggered by these same Aβ oligomers but over an extended period of exposure [60]. Structural analysis indicates that the hydrophobic C-terminal region of Aβ, derived from the transmembrane region of APP, accounts for the toxicity of Aβ due to the C-terminus looping back via a turn at residues 21-23 to form an anti-parallel beta-sheet [290], with subsequent self-association of Aβ via the anti-parallel beta sheets to form the aforementioned soluble, low- and high-molecular weight oligomers [22], not primarily the C-terminal sequence itself. While relatively high levels of Aβ have been shown to induce selective toxicity, we hypothesized that the N-terminal domain has an independent function, based on our findings that Aβ has a positive neuromodulatory role accounted for by the N-terminal domain [62]. To address this possibility, we examined the impact of peptide fragments derived from the N-terminal domain alone and in the context of Aβ neurotoxicity. Our previous work showed that a

non-toxic N-terminal fragment, found to arise endogenously from α-secretase activity in the brain, was more effective than Aβ in neuromodulation [62]. In our current work, it was found that the N-terminal peptides (N-Aβcore 10-15 and N-Aβ fragment 1-15) induced differential responses to calcium signaling, particularly in comparison to full-length Aβ which induces prolonged increases in intracellular calcium. Aβ-induced calcium dyshomeostasis likely leads to mitochondrial dysregulation as well as changes in intracellular signaling pathways and gene expression. We further hypothesized that these non-toxic N-terminal Aβ peptides would be neuroprotective against $A\beta_{1-42}$ neurotoxicity, based on our previous findings demonstrating that the N-Aβ fragment can reverse synaptic dysregulation resulting from full-length Aβ [62].

Previous evidence suggested that the neuromodulatory activity of N-Aβ fragment, and hence Aβ, resides in a core hexapeptide sequence, YEVHHQ, encompassing residues 10-15 in Aβ. Through an alanine scan, we have found the two histidine residues at positions 13 and 14 to be of key importance in activating the α7- and α4β2- nAChRs, most likely due to their specificity to the target receptors. The tyrosine at position 10 was also found to make a significant contribution to activation. As previously noted, examining the N-Aβ fragment and N-Aβcore over a prolonged time-frame also revealed qualitatively different Ca^{2+} responses in the model neuronal system, with the N-Aβcore triggering a relatively brief transient peak compared to the longer sustained Ca^{2+} enhancement induced by Aβ, consistent with previous findings [146, 284]. Additionally, studies have shown that Aβ plays a role in disrupting Ca^{2+} homeostasis by altering the activity of calcium conducting ion channels, including, notably, NMDA-type glutamate receptors [291, 292], and dramatically increasing IP_3-mediated Ca^{2+} release [293]. The variation in

Ca^{2+} responses may have been the result of differences in coupling to intracellular Ca^{2+}, consequently activating different pathways and thus causing a unique signaling cascade for each N-terminal peptide. It may also be that the differences resulted in part from differences in nAChR inactivation; however, this would best be assessed through ionic current measurements. Nonetheless, the differential cellular responses to the N-Aβcore and the N-Aβ fragment may account for the lack of neurotoxicity by these peptides.

The underlying molecular mechanism(s) responsible for the prolonged increase in intracellular Ca^{2+} caused by the neurotoxic actions of Aβ may be a homeostatic failure. Considerable evidence supports a link between mitochondrial damage and dysfunction triggered by Ca^{2+} dysregulation occuring early on in the disease. Changes in mitochondria function [26], morphology [25, 136], and dynamics [25, 294] resulting from the pathological effects of Aβ have been reported, with changes in mitochondrial bioenergetics dependent on decoding Ca^{2+} signals [295]. Here, we show that just one day of sustained Aβ exposure was enough to affect the mitochondrial membrane potential in our model system. Ca^{2+} homeostasis in the mitochondria is dependent upon the mitochondrial membrane potential, and, conversely, pathologically high levels of mitochondrial Ca^{2+} will collapse the membrane, resulting in an efflux of Ca^{2+} triggering a cell-death signaling cascade [296]. Interestingly, treatment with the N-Aβcore prevents the inactivation of the membrane potential by $Aβ_{1-42}$, further suggesting that the transient Ca^{2+} response elicited by the N-Aβcore favors an alternative non-toxic pathway. Moreover, post-treatment with the N-Aβcore attenuated the $Aβ_{1-42}$-triggered prolonged increase in intracellular calcium, further supporting its neuroprotective action against calcium dysregulation leading to mitochondrial dysfunction.

As we had previously shown that that the N-Aβ fragment enhances synaptic regulation and fear memory, while reversing Aβ-linked inhibition of LTP [62], we subsequently addressed whether the N-Aβ fragment and N-Aβcore were capable of protecting against $A\beta_{1-42}$-induced neuronal toxicity *in vitro.* Continuous co-treatment with the N-Aβcore effectively and potently protected against $A\beta_{1-42}$ -induced oxidative stress and apoptosis. Remarkably, treatment with the N-Aβcore *after* the induction of oxidative stress by $A\beta_{1-42}$ also rescued the cells from this neurotoxicity. Furthermore, the fragment or N-Aβcore were also protective against neurotoxicity induced by high concentrations of $A\beta_{1-42}$ in the absence of sensitizing nAChRs [136], again either with co-treatment or post-treatment rescue. Together, these findings suggest that the N-Aβ fragment and N-Aβcore may be neuroprotective through altered binding of $A\beta_{1-42}$ for key target sites and/or activation of alternative pathways, such as an anti-apoptotic pathway and/or anti-oxidative pathway, blocking or reversing the process by which $A\beta_{1-42}$ induces neurotoxicity.

Consistent with previous findings [297], we found the histidine residues in the N-terminal peptides to also be of essential importance in neuroprotection, indicating a necessary role in interaction with target Aβ receptors. While the N-Aβcore was found to dock into the ligand binding site of the nAChRs [61], it will be important to examine the nature of the interaction of the N-Aβcore with other Aβ target receptors, including, in particular, cellular prion and NMDA receptors. On the other hand, the rescue by the N-Aβcore (and N-Aβ fragment) long after initiation of the toxicity program by full-length Aβ would strongly suggest that the neuroprotective action of these peptides in cellular toxicity, and potentially synaptic and behavioral dysfunction, also includes altered intracellular signaling.

As a first approach to N-Aβcore modification as a means to reduce proteolysis of the hexapeptide *in vivo*, a dually N-terminus acetylated and C-terminus amidated analog (termed “capped” N-Aβcore) was synthesized and tested for neuromodulatory and neuroprotective activities, both of which were retained with the capped version. Additional stabilization of the N-Aβcore by converting each amino acid in the sequence from the *L*-enantiomer to the *D*-enantiomer also, surprisingly, retained receptor activity. Although the direct impact of enantiomeric conversion on interaction with target receptors remains to be determined, it has been shown that short synthetic *D*-amino acid peptides are capable of mimicking *L*-amino acid counterparts in binding to and activating target receptors [298] as well as antagonizing them [e.g. [299], suggesting that ligand chirality does not necessarily affect the binding specificity of some receptors, unlike that expected for enzymes. To assess the issue directly, it would be important to understand how the receptor interacts with the peptide’s side chains and backbone. Considering the evidence for a lack of enantiomer discrimination, we postulate that the N-Aβcore interaction with the target receptor is independent of the peptide backbone, but rather its side chains, particularly the aromatic rings of the two histidines, are key and may interact with the receptor in more than one orientation. This possibility may be addressed via molecular modeling. In addition, that the N-Aβcore analogs are actually stabilized against proteolysis may be assessed using a serum stabilization assay (see [300]).

CHAPTER 3: CHARACTERIZING THE NEUROPROTECTIVE EFFECTS OF THE N-AβCORE ON Aβ-INDUCED SYNAPTIC DYSFUNCTION AND ELUCIDATING THE NEUROPROTECTIVE PATHWAY OF THE N-AβCORE

3.1 Introduction

AD is clinically characterized by changes in behavior and impairments in cognitive memory and function. Loss of critical pre- and post-synaptic markers have been reported for postmortem AD brain tissue [30, 301], suggesting that AD-related cognitive impairments are based, in large part, to synaptic dysfunction and loss. Synapses are important for cell-to-cell communication and are typically damaged in neurodegenerative disease. Additionally, accumulating evidence shows a strong link between excess soluble oligomeric Aβ and synaptic dysfunction in AD [20, 21, 145]. Cognitive decline and synaptic plasticity deficits are reported to occur prior to the accumulation of Aβ plaques and tau neurofibrillary tangles [302], supporting the idea that synaptic dysfunction and mild cognitive impairment are early events driven by soluble oligomeric Aβ rising to abnormally high levels years prior to AD diagnosis. Subsequent accumulation of plaques, and particularly, neurofibrillary tangles correlates with actual synapse and neuron loss.

Synaptic degeneration and dysfunction leads to abnormal synaptic transmission and impaired long-term potentiation (LTP) and/or long-term depression (LTD), which are important in synaptic plasticity and learning and memory paradigms. Pathological levels (high nM to μM) of Aβ have been shown to inhibit LTP-induction [20, 303, 304] and enhance LTD [305, 306] in the hippocampus. On the other hand, low physiological levels

(pM) of Aβ was found to enhance LTP and memory, indicating a hormetic effect of Aβ on synaptic plasticity [32, 307].

NMDA-type and AMPA-type glutamate receptor activation plays an essential role in inducing LTP or LTD, depending on the levels of free cytosolic Ca^{2+} and its activation of downstream pathways [308]. Both NMDA and AMPA receptors are ionotropic and are permeable to Na^{+}, K^{+}, and, to varying degrees, Ca^{2+}. Although they have distinct physiological properties, they often coexist at the same synapse. Activation of GluR2-containing AMPA receptors induces a strong influx of Na^{+} and a small efflux of K^{+}, the net effect being depolarization of the postsynaptic neuron. Stimulation at the presynaptic neuron releases glutamate that binds to both NMDA and AMPA receptors on the postsynaptic side, but only AMPA receptors are activated during a weak stimulation resulting in a small depolarization (-35mV). At the resting membrane potential (-70mV) or during a weak depolarization, very few ions flow through the NMDA receptors, due to a magnesium block of the ion pore, and under these conditions, the field excitatory post-synaptic potentials (fEPSPs) are solely due to AMPA receptor activation. Alternatively, with a strong stimulation, AMPA receptors can efficiently depolarize the postsynaptic membrane, which, in turn, dispels the magnesium block, allowing for the influx of Na^{+} and Ca^{2+} through the NMDA receptor, and subsequently, activation of various Ca^{2+}-dependent signaling cascades that are responsible for inducing long-term synaptic changes.

Proper functioning NMDA and AMPA receptors are crucial for learning and memory, whereas improper activation can exacerbate AD pathogenesis. In addition to mediating Aβ-induced excitotoxicity, as previously mentioned, NMDA-evoked currents

can be depressed by Aβ (at high concentrations)[309], inducing LTD [144], as consequence of subsequent downstream AMPA receptor internalization [144], desensitization [310], and dendritic spine loss.

One of the most studied mechanisms but not the sole mechanism of LTP induction is the activation of CaMKII. In short, the influx of Ca^{2+} binds to calmodulin, which then binds to and activates CaMKII. This ultimately leads to the phosphorylation of various proteins, including AMPA receptors [311], and subsequently, an increase in conductance [312]. Additionally, CaMKII-mediated phosphorylation of AMPA receptors promotes translocation of AMPA receptors to the postsynaptic membrane [313]. On the other hand, LTD involves the activation of protein phosphatases, such as calcineurin and protein phosphatase 1(PP1) [231]. Although the exact mechanisms of LTD are not as well understood, it is believed to be influenced by AMPA receptor dephosphorylation and internalization [144].

The cAMP response element-binding protein (CREB) is an important transcription factor in mediating memory consolidation and LTP induction [314], neuronal survival [101] and neuroprotection [147]. Additionally, CREB is believed to upregulate the brain-derived neurotrophic factor (BDNF), which is important in modulating synaptic plasticity [148], and a neurotrophin target receptor, the tropomyosin receptor kinase receptor B (TrkB) [149], to promote cell survival. Alternatively, under pathological conditions, CREB has been shown to be downregulated in hippocampal neurons in AD models [105], thus affecting synaptic plasticity and memory formation.

All MAPK pathways, i.e. c-Jun N-terminal kinases (JNKs), extracellular signal regulated protein kinases (ERKs) and p38 have been implicated in Aβ pathogenesis [136,

256]: mediating tau hyperphosphorylation [315, 316], inducing apoptosis [317, 318], and activating γ- and β-secretases [319, 320]. Importantly, the ERK cascade has also been shown to play a role in learning and memory [242, 321] by activating important transcription factors implicated in synaptic plasticity [322], upregulating the translation of proteins associated with LTP [323], and increasing AMPA receptor insertion into postsynaptic membrane [324]. The exact mechanism in which Aβ induces LTP deficits is not well understood, but studies have indicated a potential link of the activation of certain kinases, specifically JNK and p38, to Aβ-induced LTP and synaptic dysfunction [244, 325].

We have shown that at low concentration (pM-nM) the N-terminal Aβ fragment comprising amino acids 1-15/16 (N-Aβ fragment) is nearly twice as effective as full-length Aβ as a neuromodulator, stimulating receptor-linked increases in neuronal Ca^{2+}, enhancing synaptic plasticity and enhancing contextual fear memory [62]. Considering the evidence that the N-Aβcore is the active region of the N-Aβ fragment, we aimed to better understand the neuroprotective mechanism of the N-Aβcore on synaptic plasticity. We assessed whether the N-Aβcore could rescue LTP and LTD dysfunction resulting from prolonged, elevated levels of Aβ, while aiming to elucidate the neuroprotective mechanism of the N-Aβcore against Aβ-neurotoxicity and synaptic deficits.

3.2 Methods

3.2.1 Animals

All animal handling, surgery, use and euthanasia were performed under an approved IACUC protocol (11-1219-6 or 16-2282-2), compliant with NIH and Society for Neuroscience guidelines for use of vertebrate animals in neuroscience research. The human APP/PSEN1 mouse line, 5XFAD (Tg6799), on the B6.SJL background (B6SJL-Tg(APPSwFlLOn,PSEN1*M146L*L286V) 6799Vas/Mmjax; obtained from JAX stock #006554, MMRRC034840 Hemizygous) was used as a well characterized model for Aβ-based pathology and neurodegeneration [326], along with age-matched control (B6.SJL background) mice (MMRRC034840 Non-carrier). Mice at 7- to 8-months of age of both sexes (weight range: 28-35 g), originally housed in ventilated enrichment cages in the John A. Burns School of Medicine AAALAC-accredited Vivarium with *ad libitum* access to food and water, were used in roughly equal numbers, this age range selected for displaying pronounced LTP deficits in the transgenic line. Inclusion/exclusion criteria were based on animal health.

3.2.2 Extracellular field potential recordings in hippocampal slices

Hippocampal slices were prepared from 7- to 8- month-old 5XFAD (Tg6799) or B6.SJL (control mice). Cervical dislocation and decapitation were performed under an approved IACUC protocol (11-1219-6 or 16-2282-2). Brains were extracted into ice-cold artificial cerebral spinal fluid (aCSF) consisting of 130 mM NaCl, 3.5 mM KCl, 10 mM glucose, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.5 mM $MgSO_4$, and 24 mM $NaHCO_3$, bubbled with 95% O_2/5% CO_2. Transverse brain slices of 400μm were obtained using a

Leica vibrating microtome (Leica, VT1200S) and quickly transferred to fresh ice-cold aCSF for hippocampi isolation. Extracted hippocampi slices were incubated in bubbled aCSF in a holding chamber for 30 min at room temperature (23°C) after which the holding chamber was transferred to a 32°C water bath for 1 h. The chamber was then removed from the water bath and placed at room temperature for another 1 h prior to recording. The slices were subsequently transferred to a recording chamber and perfused at 3mL/min with aCSF (bubbled with 95% O_2/5% CO_2) at 32°C. The Schaffer collateral fibers were stimulated at a frequency 0.1 Hz using a bipolar stimulating electrode and CA1 field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass electrode filled with 3M NaCl (resistance 1-1.5 MΩ). Basal synaptic transmission was assessed by comparing stimulus strength against fEPSP slope to generate input/output (I/O) curves. In the following experiments, a minimum of 20 min baseline stimulation was performed, recording every minute. The baseline and stimulus current were adjusted during this period so that fEPSP stabilized at 30-40% of maximum.

LTP was induced by a 3-theta-burst stimulation (TBS) protocol, where each burst consisted of 4 pulses at 100 Hz with a 200-ms interburst interval. LTD was induced using a low frequency stimulation (LFS) protocol, consisting of a 1Hz single pulse stimulus (900 pulses for 15 min). TBS and LFS were administered after a 20-min baseline recording period for aCSF alone or a 35-min baseline recording period for aCSF (15 min) followed by N-Aβcore (20 min). Importantly, TBS and LFS were administered in the presence of the N-Aβcore for treatment experiments.

3.2.3 Protein extraction

NG108-15 cells were cultured as described in section 2.2.1. The NG108-15 cells were allowed to grow to 80% confluency in a 25 cm^2 flask prior to differentiation (described in section 2.2.1) and subsequent transfection (described in section 2.2.1) 72 h later. Forty-eight hours after transfection, NG108-15 cultures were subjected to various treatments for three days. The medium for each treatment condition was changed every day, unless otherwise noted.

For protein extraction, the cells were washed with cold 1x phosphate-buffered saline (PBS) and lysed with 200 μL of 1% SDS (Fisher Scientific, catalog # BP166-100, lot 122975). The lysates were frozen at 20°C and immediately transferred to a 95°C heating block for 5-10 min, followed by sonication for 15-20 min to shear the DNA. The total amount of protein was quantified by a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, catalog # 23225).

3.2.4 Western blot

Gel sample buffer (4x; ThermoFisher Scientific, catalog # B0007, lot # 1549819) and reducing agent (10x; ThermoFisher Scientific, catalog # B0009, lot # 1901009) were added to diluted SDS-solubilized protein samples for a final protein concentration of 1 μg/μL. The samples were boiled to 95°C for 5 min, immediately cooled on ice and then centrifuged. Equal amounts of protein were subjected to electrophoretic separation on a 4-20% Tris-Glycine polyacrylamide gel (ThermoFisher Scientific, catalog XP04200), transferred to an activated PVDF membrane (“blot”; Millipore, catalog # IPFL00010) for 2 h at 50 V, and incubated in primary antibody overnight at 4°C. Blots were washed 3x (5

min each wash) in 0.1% Tween-20 in TBS (Tris Buffered Saline) and incubated in the appropriate IR-dye conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE) for 1h. An Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE) was used for detection and analysis was performed via Image Studio v4.0 software (LI-COR Biosciences, Lincoln, NE).

3.2.3 Antibodies

The following primary antibodies were used for detection. Rabbit anti-phospho CREB at 1:1000 dilution (Cell Signaling, catalog # 9198S, lot 14), rabbit anti-CREB at 1:1000 dilution (Cell Signaling, catalog # 9197S, lot 16), rabbit anti-phospho PERK at 1:1000 dilution (Cell Signaling, catalog # 3179S), rabbit anti-PERK at 1:1000 dilution (Cell Signaling, catalog #3192S), mouse anti-phospho JNK at 1:500 dilution (Santa Cruz Biotechnology, catalog # 6254, lot G0711), rabbit anti-SAPK/JNK at 1:1000 dilution (Cell Signaling, catalog # 9252S, lot 16), rabbit anti-phospho p44/42 MAPK at 1:1000 dilution (Cell Signaling, catalog # 4370S, lot 12), mouse anti-p44/42 MAPK at 1:1000 dilution (Cell Signaling, catalog # 4696S, lot 21), mouse anti-β-actin at 1:2000 dilution (Sigma Aldrich, catalog # A2228, lot 112M4762V), and mouse anti-β-tubulin at 1:1000 dilution (Cell Signaling, catalog # 86298S, lot 1).

3.2.4 Reactive Oxygen Species (ROS)/ Hoechst staining

See section 2.2.4.

3.2.5 Statistical analysis

Treatment and units were randomized as to order for all assays and experiments. Biological replicates were based on independent samples (*n*). All experiments were repeated at least three times unless otherwise noted. Multiple comparisons were made using one-way ANOVA with Bonferroni or Tukey post hoc tests, as indicated. Paired comparison was made using Student's *t*-tests. *P*-values <0.05 were considered the minimum for significance (as rejection of the null hypothesis). Unless otherwise noted, data were analyzed and graphed with GraphPad Prism 5 (GraphPad v5.0b; RRID:SCR_002798) using the appropriate statistical tests.

3.3 Results

3.3.1 The N-Aβcore protects against LTP deficits induced by pathological levels of full-length Aβ

We have previously shown that the N-Aβ fragment enhances synaptic plasticity and contextual fear memory while protecting against Aβ-linked synaptic impairment [62]. Considering the evidence that the N-Aβcore accounts for the neuromodulatory activity of the N-Aβ fragment, we assessed whether the N-Aβcore is capable of protecting against Aβ-induced synaptic dysfunction *ex vivo*. We utilized hippocampal slices from our APP (Aβ) mouse model (5xFAD) and their wild-type counterparts (B6.SJL) to examine synaptic transmission. Basal synaptic transmission at the Schaffer collateral-CA1 synapses (input-output curve) shows that the fEPSP slopes versus stimulus strength for both the 5xFAD and B6.SJL mice were comparable (Fig. 12A), ruling out any issues with regard to baseline synaptic strengths. Interestingly, treatment with the N-Aβcore during baseline

recordings shows an increase in baseline synaptic transmissions for both 5xFAD and B6.SJL but was only significant in the B6.SJL slices (Fig. 12B). To assess sustained changes in synaptic plasticity, we used a 3x TBS stimulation protocol at the Schaffer collaterals to measure LTP (see cross-sectional cartoon of the hippocampus in Fig. 12A, inset). LTP showed a trend toward enhancement for the N-Aβcore-treated B6.SJL slices, though it was not significant (Fig. 12C). Consistent with previous findings, LTP in the 5xFAD slices was substantially reduced compared to that observed for slices from B6.SJL [327] (Fig. 12C), dropping to near baseline at 60 min post-TBS. By contrast, treatment with 500nM N-Aβcore restored LTP in the 5xFAD slices to the level seen for the wild-type slices (Fig. 12C and 12D). These findings demonstrate that the N-Aβcore can reverse LTP deficits induced from prolonged exposure to pathological levels of Aβ, while enhancing basal synaptic transmission.

Through a receptor-linked Ca^{2+} assay, we have shown that mutating the tyrosine residue in the N-Aβcore to a serine [Y10S] or mutating the two histidine residues to two alanines [H13A,H14A] reduces activity (Fig. 4), indicating these amino acid residues in the N-Aβcore sequence are essential for activity. As a control, we assessed synaptic changes with the reverse N-Aβcore [QHHVEY] and a triple (inactive) mutant [SEVAAQ] on LTP in 5xFAD hippocampal slices. Surprisingly, treatment with the reverse N-Aβcore potentiated LTP in the 5xFAD hippocampal slices (Figs. 13A and 13B). By contrast, the inactive triple mutant had no effect on synaptic potentiation in the 5xFAD slices (Figs. 13A and 13B). It is important to note that we did not see an increase in basal synaptic transmission or a trend toward increasing LTP in the wild-type slices with the reverse N-Aβcore, as seen for the N-Aβcore (Fig. 12). Taken together, these results suggest that

the two histidine residues, in particular, contribute to the strong positive neuromodulatory activity of the N-Aβcore.

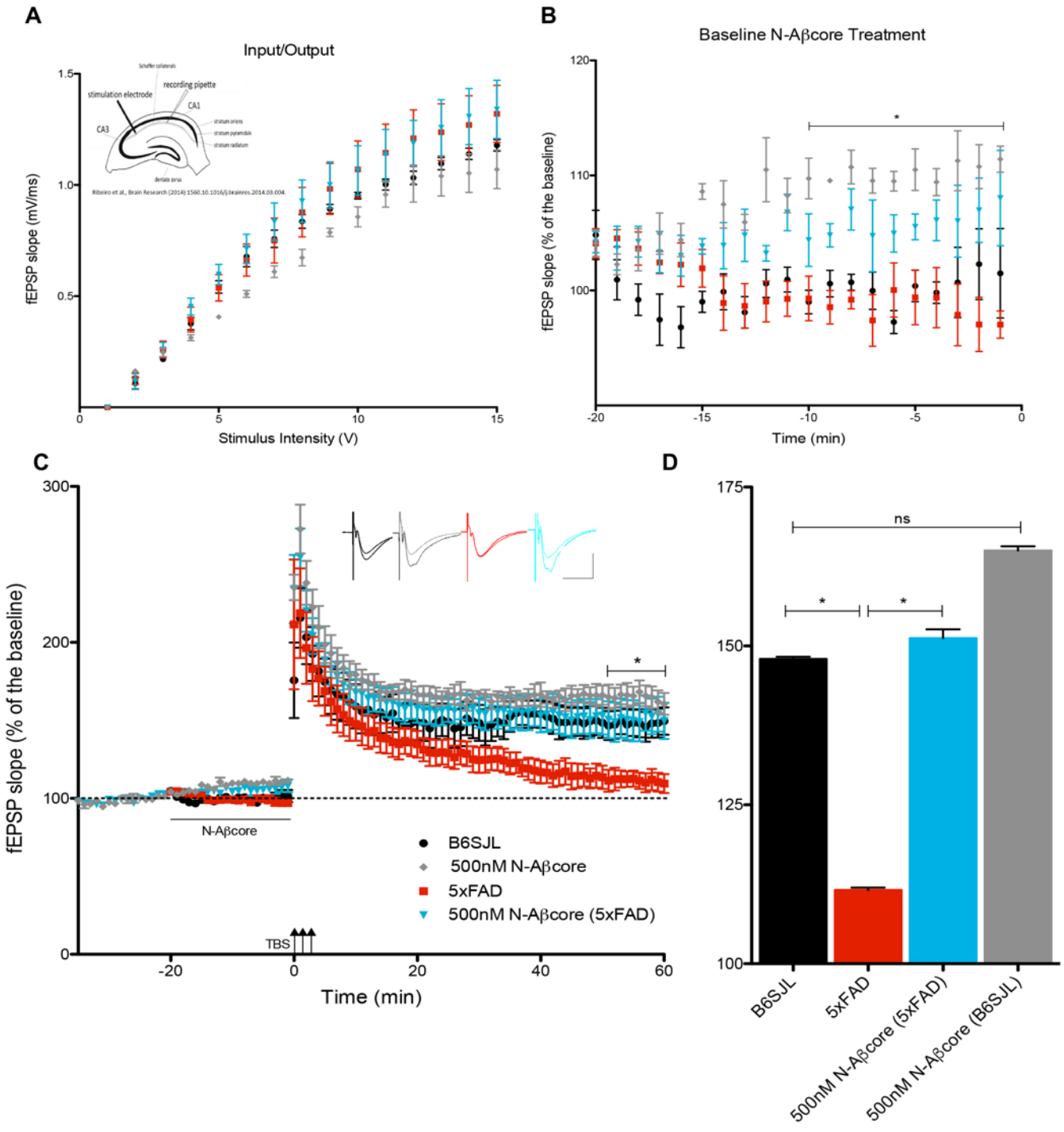


Figure 12: The N-Aβcore protects against Aβ-induced synaptic impairments

Sustained changes on synaptic plasticity by a 3x-TBS protocol. A, Input/Output curve plotting fEPSP slopes verses stimulus strength before treatment. B, Baseline recordings prior to stimulation treated with control aCSF in 5xFAD (red, n=4) and B6.SJL (black, n=5) or 500nM N-Aβcore in 5xFAD (blue, n=4) and B6.SJL (grey, n=3). C, TBS-induced LTP, with color-coded inserts showing examples of fEPSP for B6.SJL perfused with control aCSF (black, n=5) or 500nM N-Aβcore (grey, n=3) and 5xFAD with aCSF (red, n=4) or 500nM N-Aβcore (blue, n=4). D, Average fEPSP slope values for 50-60 min post-tetanus. n refers to the number of slices. Data are means ± S.E.M. C, inset calibration: horizontal, 10ms; vertical, 0.4 mV. *p<0.05 (Bonferroni *post hoc* tests for comparison with 5xFAD aCSF treatment (red)).

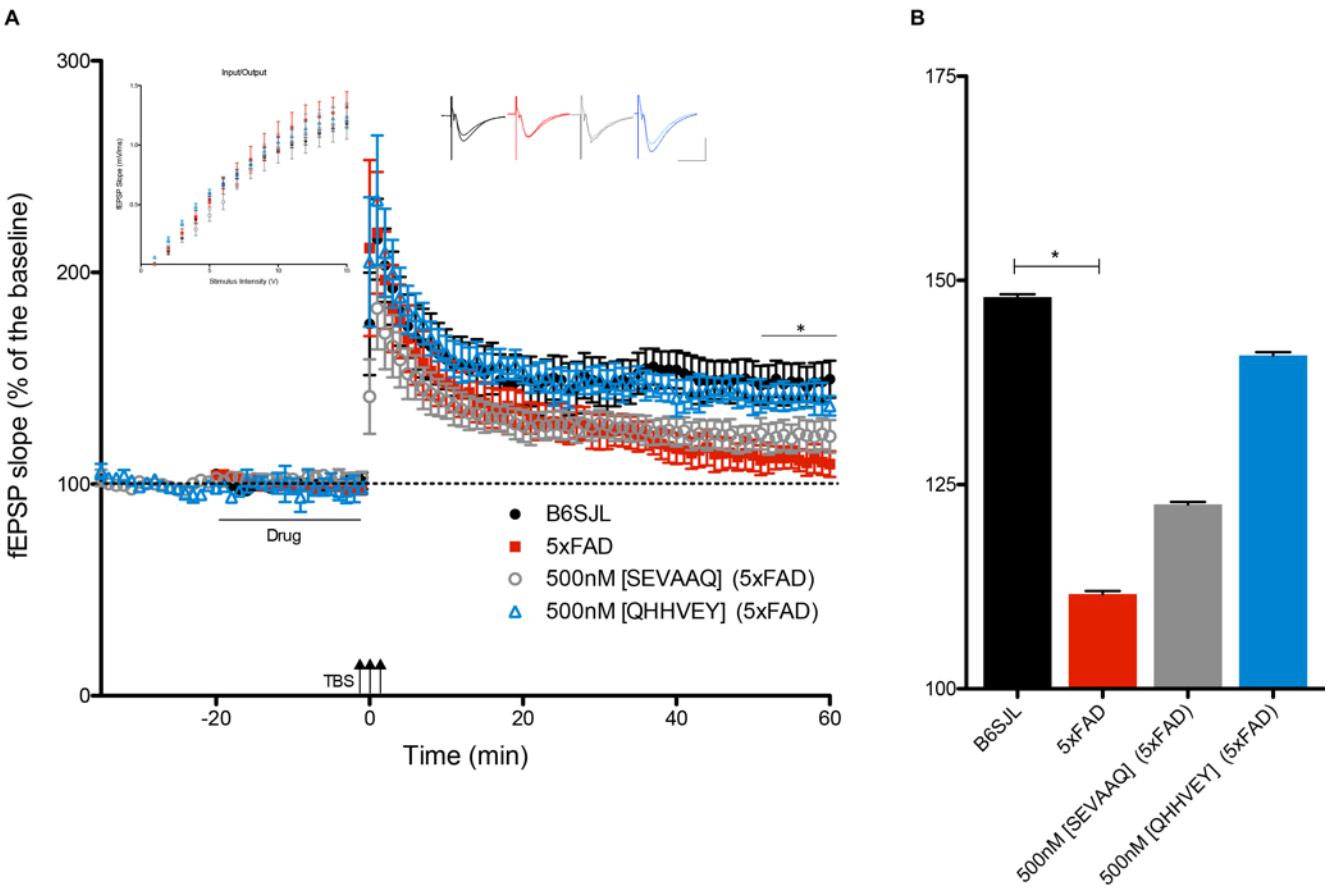


Figure 13: The reverse N-Aβcore and inactive triple mutant on LTP

Sustained changes on synaptic plasticity by a 3x TBS protocol. A, TBS-induced LTP, with control input/output and color-coded inserts showing examples of fEPSP for B6.SJL perfused with control aCSF (black, n=5) and 5xFAD with aCSF (red, n=4), 500nM [SEVAAQ] (grey, n=5) or 500nM [QHHVEY] (blue, n=3). B, Average fEPSP slope values for 50-60 min post-tetanus. n refers to the number of slices. Data are means ± S.E.M. A, inset calibration: horizontal, 10ms; vertical, 0.4 mV. *p<0.05 (Bonferroni *post hoc* tests for comparison with B6.SJL aCSF treatment (black)).

We further examined different concentrations of the N-Aβcore on reversing Aβ-induced LTP deficits. As compared to the nM concentration of the N-Aβcore used previously (Figs. 12 and 13), low concentrations (fM) of the N-Aβcore showed no difference on LTP compared to control 5xFAD slices (Figs. 14A and 14B). These data indicate that the reversal of Aβ-induced synaptic dysfunction by the N-Aβcore is concentration-dependent. We therefore postulate that the rescue of Aβ-linked LTP deficits by the N-Aβcore will fall within the range of fM and nM.

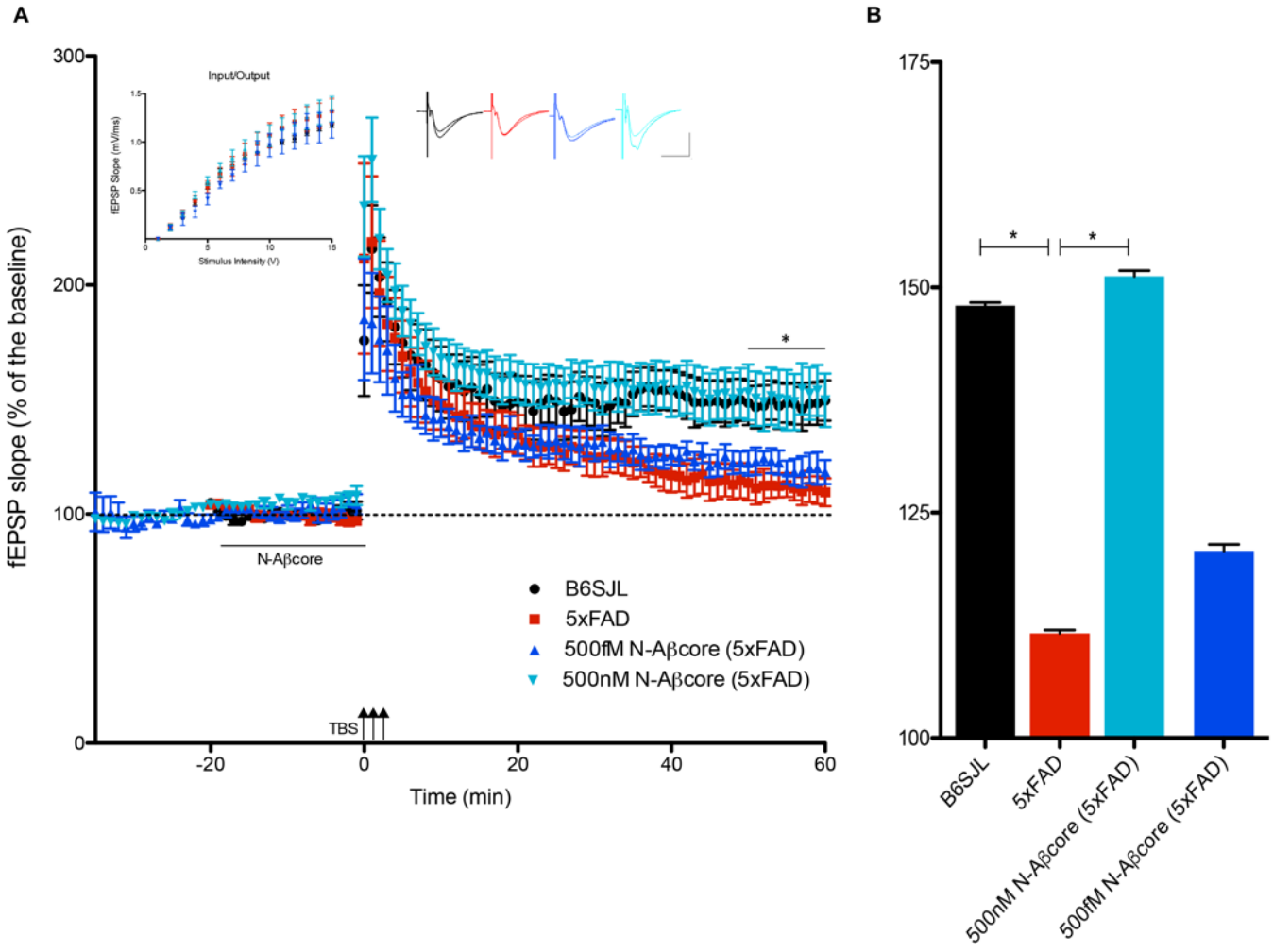


Figure 14: The N-Aβcore protection of Aβ-induced synaptic dysfunction is concentration dependent

Sustained changes on synaptic plasticity by a 3x TBS protocol. A TBS-induced LTP, with control input/output and color-coded inserts showing examples of fEPSP for B6.SJL perfused with control aCSF (black, n=5) and 5xFAD with aCSF (red, n=4), 500fM N-Aβcore (dark blue, n=3) or 500nM N-Aβcore (light blue, n=4). B Average fEPSP slope values for 50-60 min post-tetanus. n refers to the number of slices. Data are means ± S.E.M. Calibration: horizontal, 10ms; vertical, 0.4 mV. *p<0.05 (Bonferroni post hoc tests for comparison with 5xFAD aCSF treatment (red)).

3.3.2 Elevated levels of Aβ enhances long-term depression and the N-Aβcore reverses Aβ-induced LTD enhancement

Another important aspect of synaptic plasticity is LTD and to date, very few studies have examined the effects of pathological levels of soluble Aβ on LTD induction, and moreover, the results have been inconsistent. For example, focusing on NMDA receptor-dependent LTD, the administration of synthetic Aβ resulted in an enhancement of LTD [306, 328] whereas similar studies conducted by other groups show no effect [329]. Here we aimed to examine the effects of endogenous soluble Aβ on LTD. Using LFS to induce LTD in the Schaffer collateral – CA1 pathway, LTD in slices from 5xFAD mice was significantly enhanced compared to that observed for LTD induced in slices from B6.SJL control mice (Figs. 15A and 15B). Interestingly, treatment with the N-Aβcore prior to and during LFS resulted in a near-complete rescue of LTD in the 5xFAD mouse slices (Figs. 15A and 15B). Taken together, these data suggest that Aβ plays a role in facilitating LTD and the N-Aβcore may protect against Aβ-induced LTD enhancement. The role of the NMDA receptor on Aβ facilitation of LTD warrants further investigation.

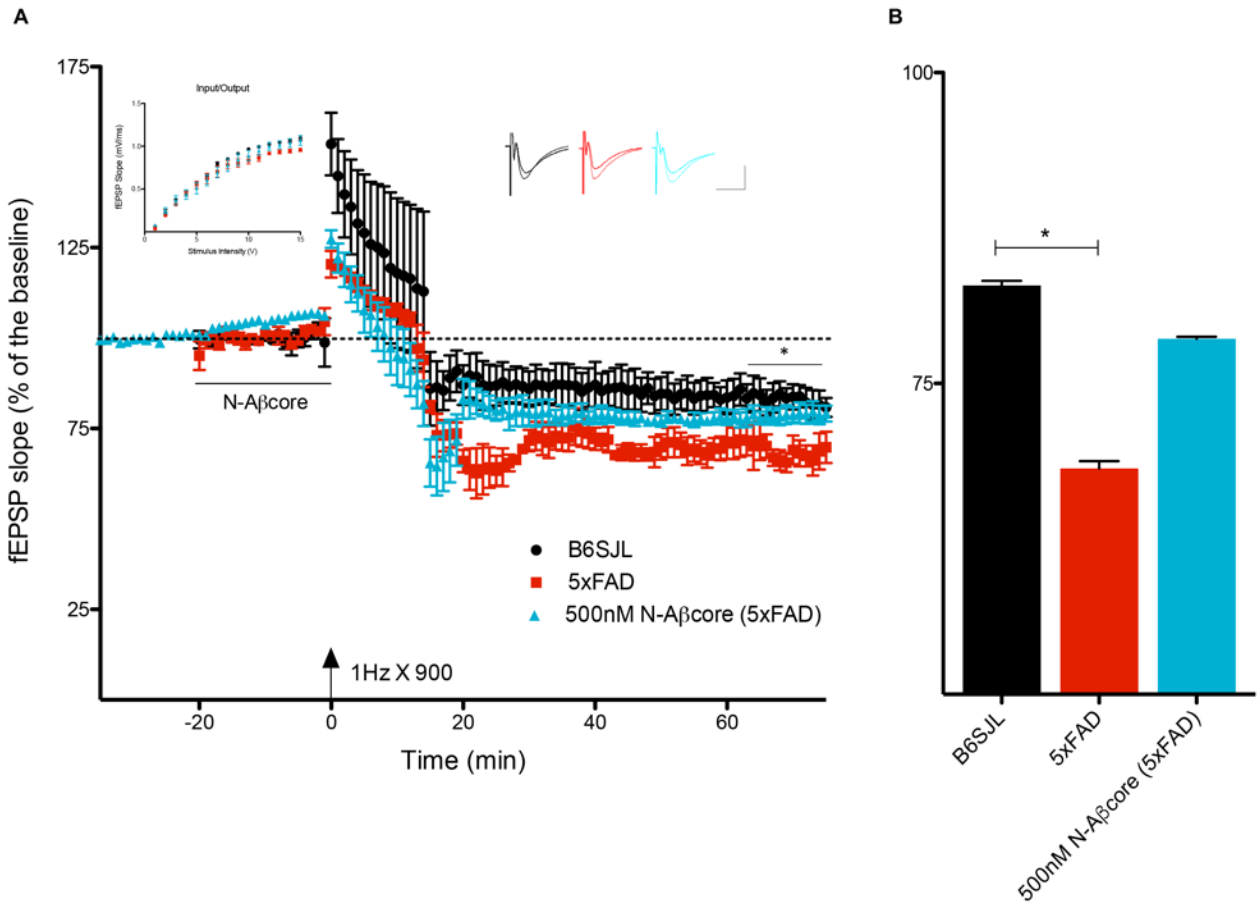


Figure 15: The N-Aβcore reverses endogenous Aβ enhancement of LTD

Sustained changes in synaptic plasticity by a 1Hz LFS protocol. A, LFS-induced LTD, with control input/output and color-coded inserts showing examples of fEPSP for B6.SJL perfused with control aCSF (black, n=3) and 5xFAD with aCSF (red, n=3), or 500nM N-Aβcore (blue, n=4). B, Average fEPSP slope values for 50-60 min post-tetanus. n refers to the number of slices. Data are means ± S.E.M. Inset scale calibration: horizontal, 10ms; vertical, 0.4 mV. *p<0.05 (Bonferroni post hoc tests for comparison with B6.SJL aCSF treatment (black)).

3.3.3 The N-Aβcore rescues Aβ-induced activation of cellular stress markers ERK and JNK

We have previously shown that prolonged exposure to nanomolar levels of Aβ activates various stress markers in the MAPK pathway while in the presence of the sensitizing α4β2-nAChRs *in vitro* [136]. In accordance with our previous studies, we show an increase in phosphorylation of ERK and JNK, indicative of activation (Figs. 16 and 17, respectively) upon nM treatment with Aβ in α4β2-nAChR-transfected NG108-15 cell cultures. Furthermore, treatment with varying concentrations of the N-Aβcore (pM and nM) trended towards a reduction in phosphorylation of ERK and JNK (Figs. 16 and 17, respectively). Surprisingly, pM concentrations of the N-Aβcore inhibits phosphorylation of JNK to below baseline levels (Fig. 17), suggesting an unusual concentration-dependent mechanism for N-Aβcore activity.

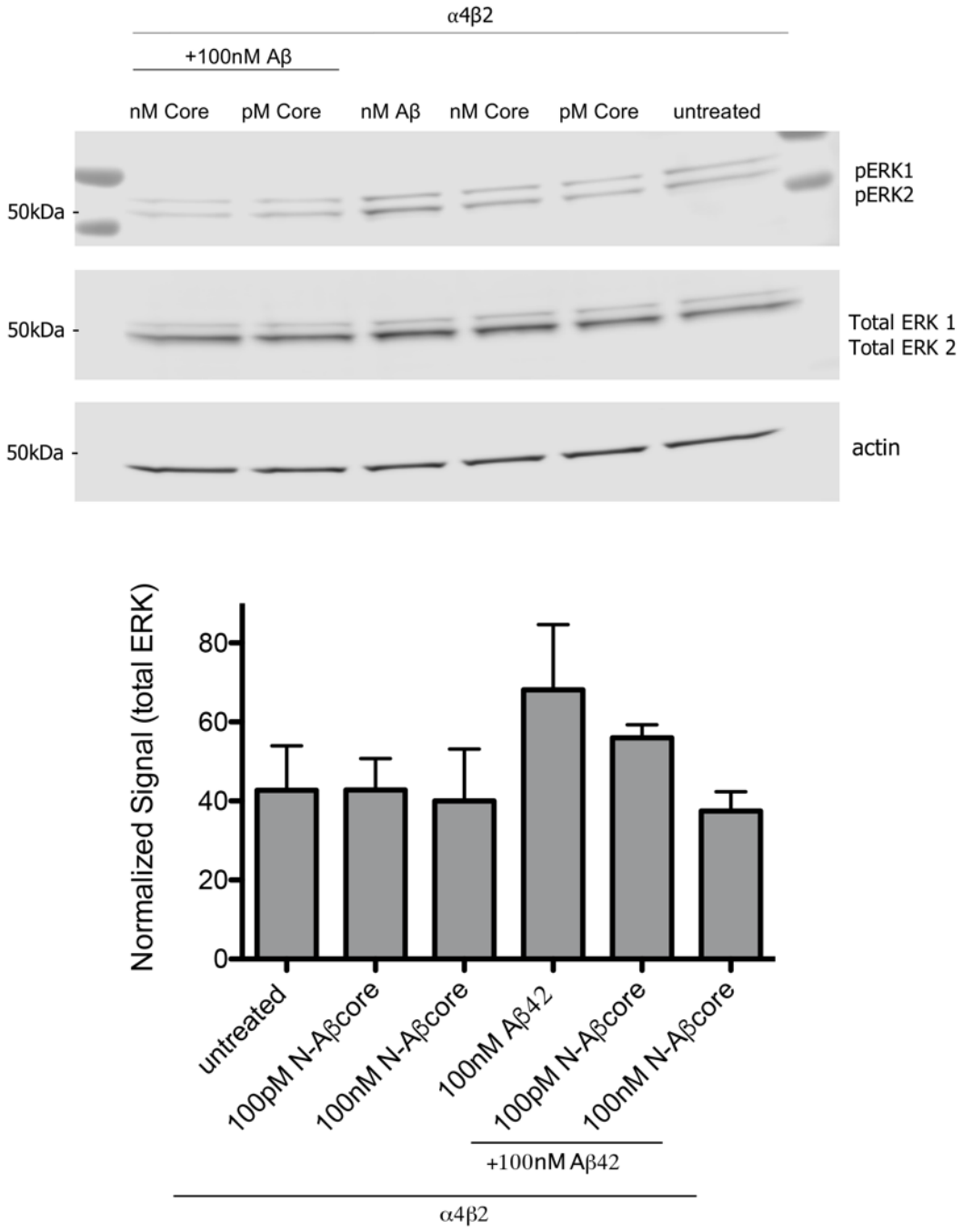


Figure 16: The N-Aβcore rescues Aβ activation of ERK

Western blot of total and phospho-ERK in protein extracts from nAChR-expressing NG108-15 cells treated with N-Aβcore, Aβ or both. (Top) Representative western immunoblot image showing levels of phopho-ERK1 and phopho-ERK2, total ERK1 and total ERK2, and actin (loading control). (Bottom) Quantification of the expression of pERK1 and pERK2 normalized to total ERK, after adjusted for protein loading. Treatment concentrations were 100pM or 100nM. n=3, n refers to number of replicates. Data are means ± S.E.M.

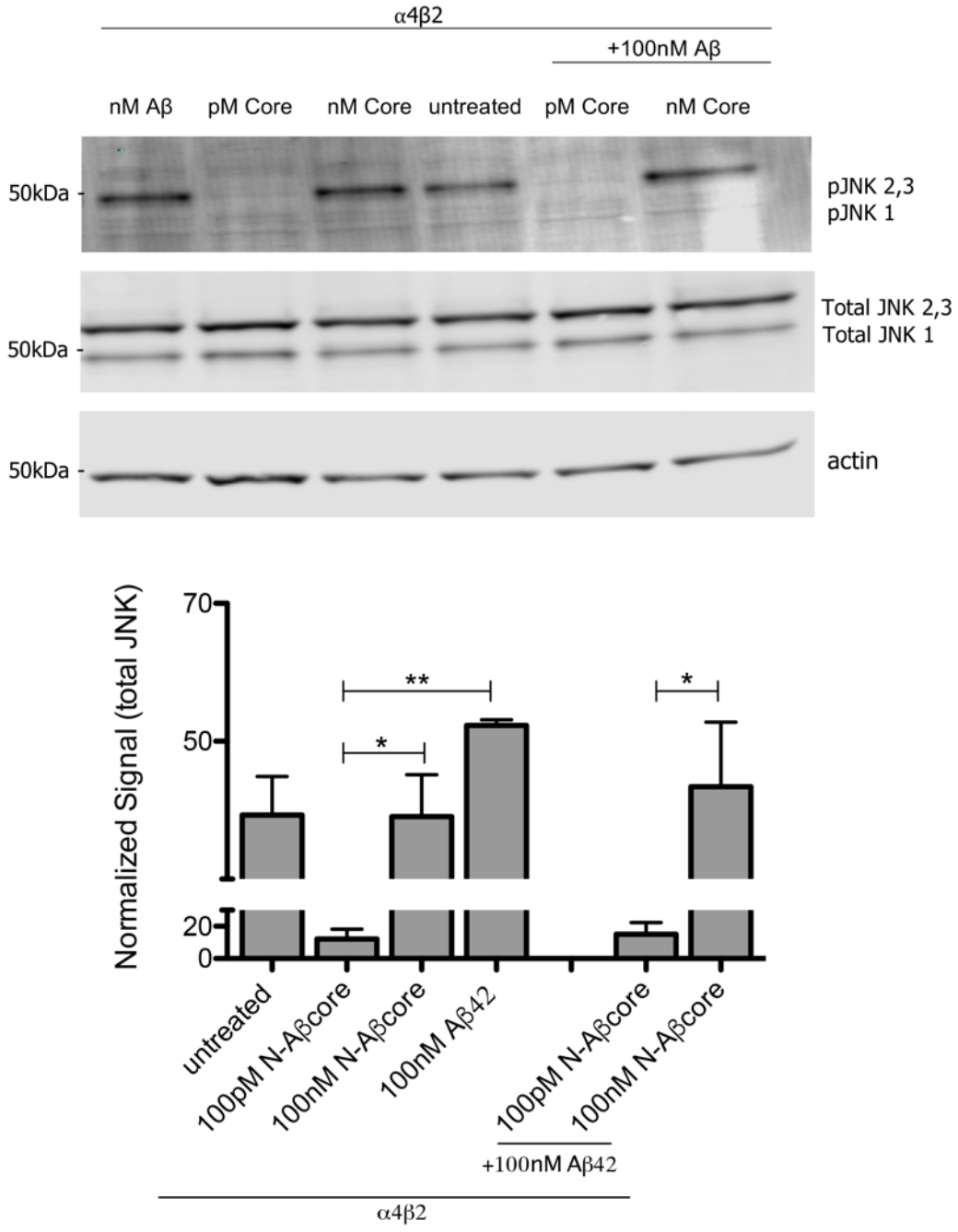
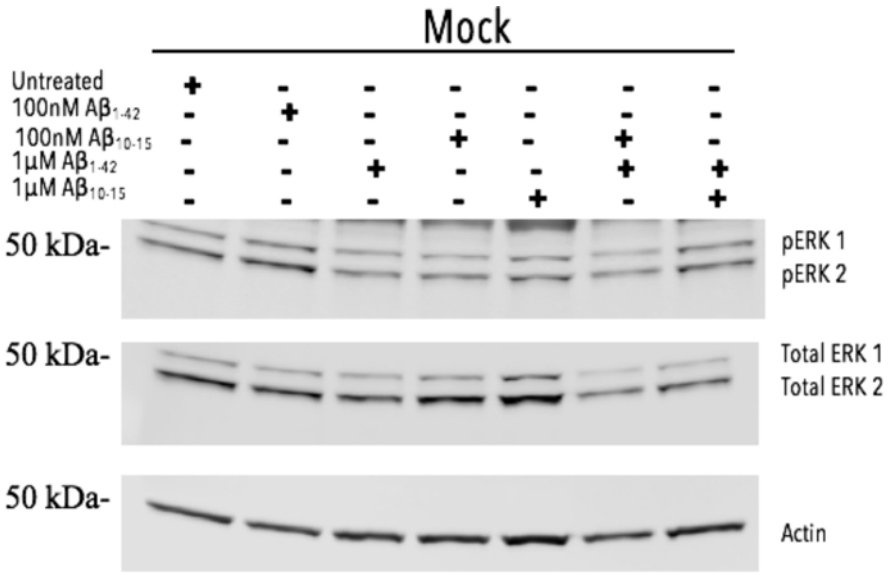


Figure 17: The N-Aβcore rescues Aβ activation of JNK

Western blot of total and phospho-JNK in protein extracts from nAChR-expressing NG108-15 cells treated with N-Aβcore, Aβ or both. (Top) Representative western immunoblot image showing levels of phopho-JNK1 and phopho-JNK2,3, total JNK1 and total JNK1,2, and actin (loading control). (Bottom) Quantification of the expression of pJNK1 normalized to total JNK after adjusting for protein load Treatment concentrations were 100pM or 100nM. n=3, n refers to number of replicates. Data are means ± S.E.M. *p<0.05, **p<0.005 (Tukey post hoc tests for comparison)

Next, we assessed whether the reduction of Aβ-induced ERK and JNK activation on treatment with the N-Aβcore was specific to α4β2-nAChRs. In the absence of α4β2-nAChRs, we found that μM concentrations of Aβ are necessary to activate ERK and JNK to above baseline levels (Figs. 18 and 19, respectively). While treatment with nM-μM N-Aβcore did not seem to reduce Aβ-induced ERK activation (Fig. 18), preliminary experiments treatment with the N-Aβcore showed a trend towards a reduction in Aβ-induced JNK activation independent of nAChRs (Fig. 19).



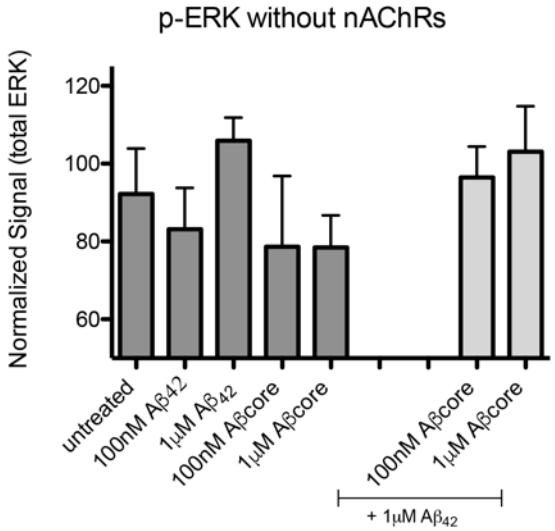


Figure 18: Aβ-induced activation of ERK in the absence of nAChRs

Western blot of total and phospho-ERK in protein extracts from nAChR-expressing NG108-15 cells treated with N-Aβcore, Aβ or both. (Top) Representative western immunoblot image showing levels of phopho-ERK1 and phopho-ERK2, total ERK1 and total ERK2, and control actin. (Bottom) Quantification of the expression of pERK1 and pERK2 normalized to total ERK. n=3, n refers to number of replicates. Data are means ± S.E.M.

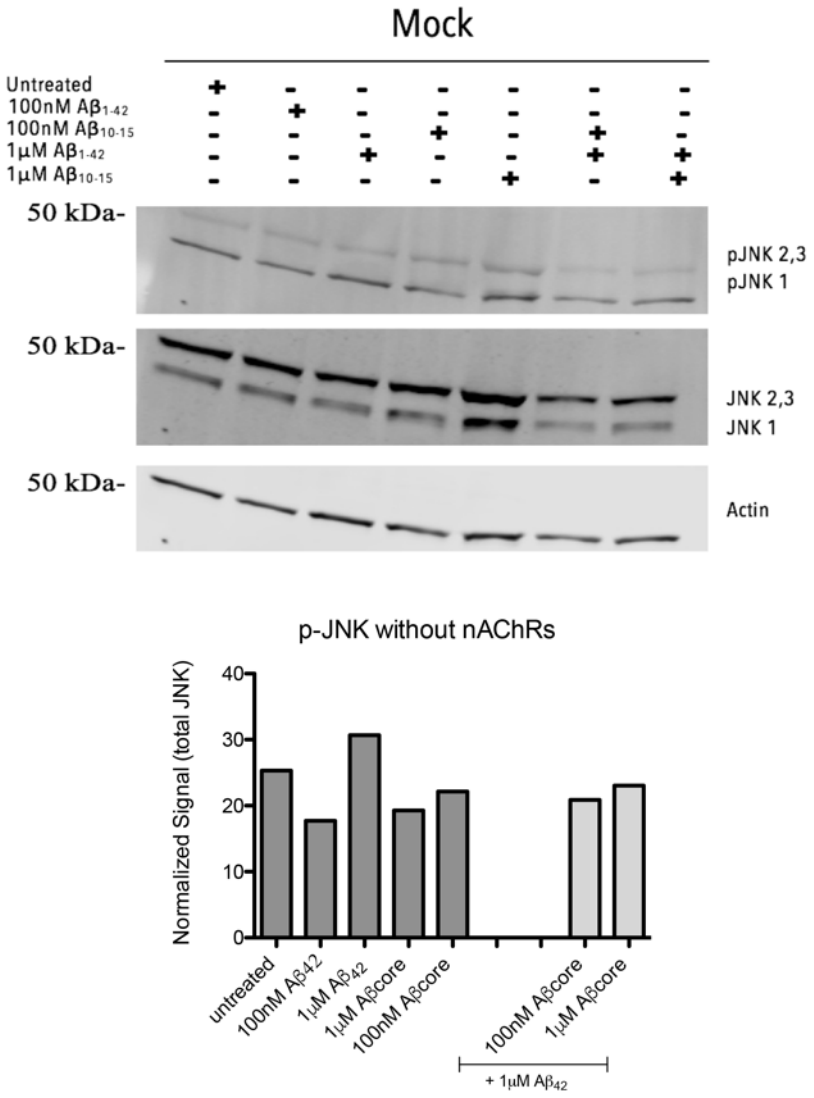


Figure 19: Aβ-induced activation of JNK in the absence of nAChRs

Western blot of JNK in protein extracts from nAChR-expressing NG108-15 cells treated with N-Aβcore, Aβ or both. (Top) Representative western immunoblot image showing levels of phopho-JNK1 and phopho-JNK2,3, total JNK1 and total JNK1,2, and control actin. (Bottom) Quantification of the expression of pJNK1 normalized to total JNK. Treatment concentrations were 100pM or 100nM. n=2, n refers to number of replicates.

Together these preliminary data indicate that the N-Aβcore may be able to protect against Aβ activation of the stress kinase JNK in both nicotinic receptor-expressing and control cells, whereas the reduction of ERK activation by the N-Aβcore appears to be nAChR-dependent.

3.3.4 Picomolar concentration of the N-Aβcore reduces reactive oxygen species levels to below baseline

Our surprising, yet interesting findings that pM levels of the N-Aβcore inhibits JNK activation (Fig. 17) prompted us to investigate oxidative stress at low N-Aβcore concentrations. In preliminary experiments in the presence of α4β2-nAChRs, pM

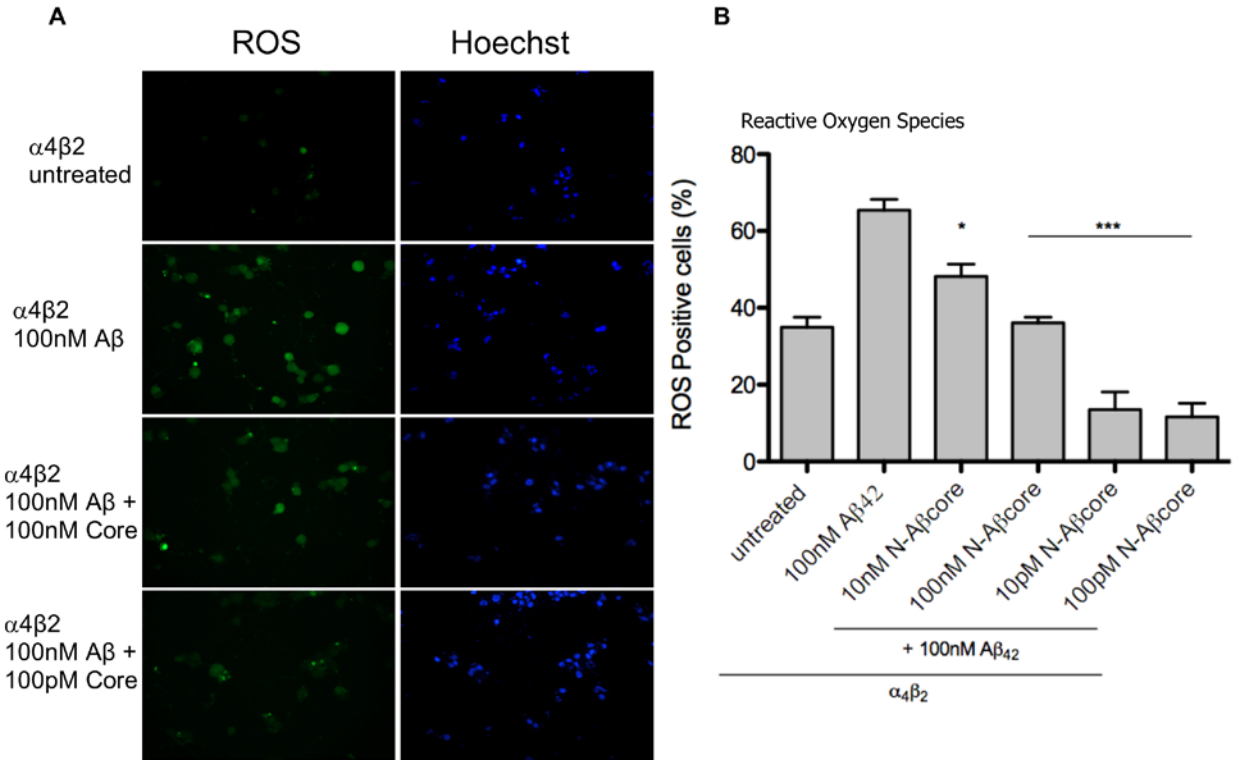


Figure 20: The N-Aβcore reduces oxidative stress levels to below baseline

A, Representative images of Hoechst and ROS staining of differentiated NG108-15 cells expressing nAChRs. B, Treatment with treatment with pM-nM N-Aβcore with 100nM $A\beta_{1-42}$ in the presence α4β2-nAChR (*n*=2). n refers to number of replicates

concentrations of the N-Aβcore not only protected against the Aβ-induced oxidative stress, but also reduced ROS levels to below baseline (Fig. 20). These data suggest that the extent of neuroprotective activity of the N-Aβcore also displays an unusual concentration-dependence and that the N-Aβcore may be eliciting an Aβ-independent, alternative neuroprotective pathway.

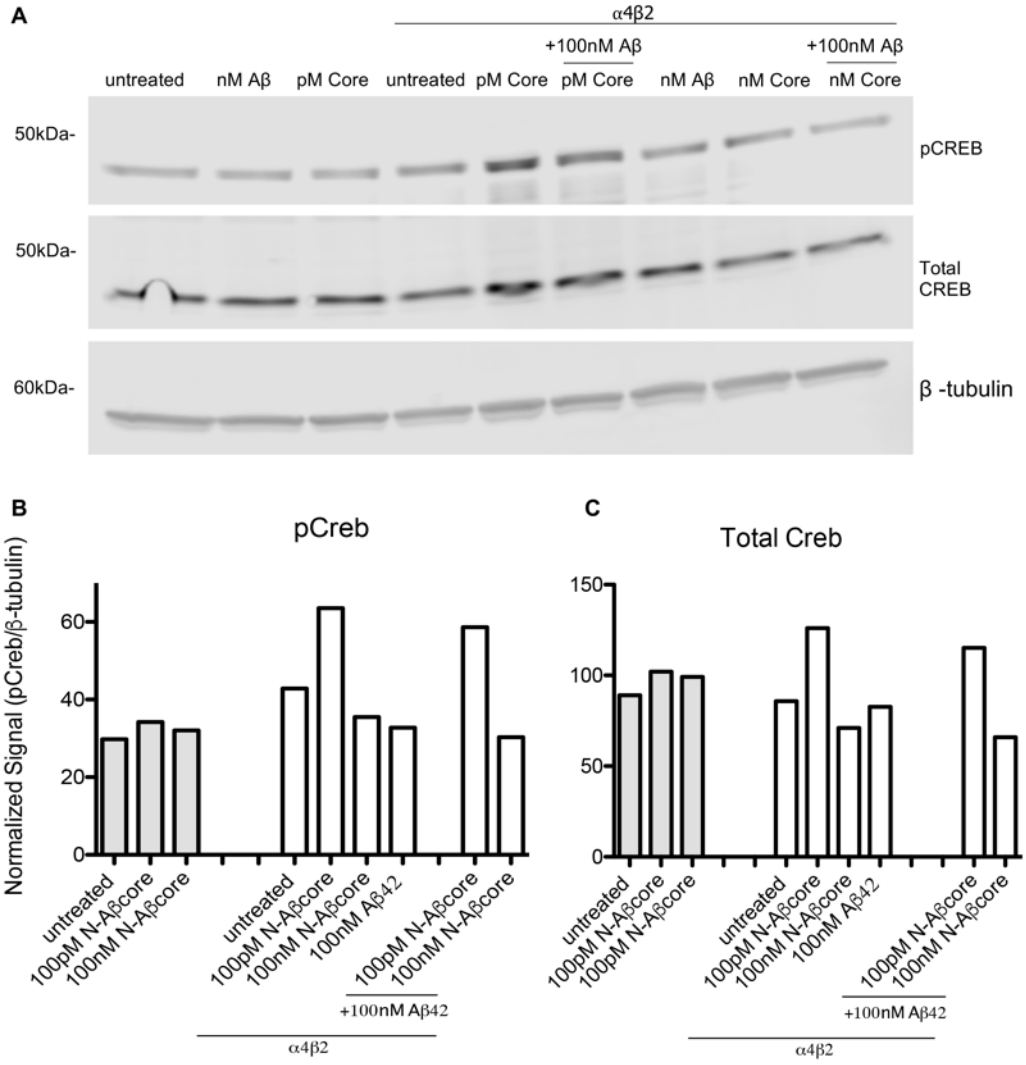


Figure 21: Low levels of the N-Aβcore upregulates CREB activity and expression

Western blot of total and phospho-CREB in protein extracts from nAChR-expressing NG108-15 cells treated with N-Aβcore, Aβ or both treatments. A, Representative western immunoblot image showing levels of pCREB, total CREB and control β-tubulin. B, Quantification of the expression of pCREB normalized to β-tubulin. C, Quantification of the expression of total CREB normalized to β-tubulin. Treatment concentrations were 100pM or 100nM. n=2, n refers to number of replicates.

3.3.5 Picomolar concentrations of N-Aβcore upregulates phospho-CREB and total CREB

CREB is an important transcription factor implicated in cell survival and proliferation as well as synaptic modulation for learning and memory (see sections 1.3.5 and 1.4.5). To better understand the neuroprotective mechanism of the N-Aβcore on cell survival and synaptic plasticity, we investigated whether the N-Aβcore affected CREB activation and expression. We found in preliminary experiments that the results with treatment with Aβ suggested a trend towards a reduction in phospho-CREB (Fig. 21B) but had no effect on total CREB levels (Fig. 21C). It is unclear whether this reduction is due solely to the presence of α4β2-nAChRs and thus warrants further investigation. Additionally, treatment with 100nM N-Aβcore showed no difference in the activation or expression of CREB compared to untreated control in both α4β2-nAChR- and mock-transfected cells (Figs. 21B and 21C) nor did it trend towards a reversal of the Aβ effect on phospho-CREB (Figs 21B). Interestingly, 100pM N-Aβcore upregulated CREB activity and expression in an α4β2-nAChR-dependent manner even in the presence of Aβ (Figs. 21B and 21C), suggesting that the N-Aβcore can directly stimulate activity. Consistent with our earlier findings, we believe that the N-Aβcore's neuroprotective action is dependent on concentration, where pM levels of the N-Aβcore have shown to be most effective. These data are generally consistent with the idea that the N-Aβcore activates an Aβ-independent neuroprotective pathway.

3.3.6 The N-Aβcore reduces Aβ-induced ER stress

Detrimental levels of Aβ induces cellular stress that effects Ca^{2+} homeostasis in the endoplasmic reticulum (ER). This further causes ER stress which, in turn, induces the accumulation of unfolded proteins, eliciting the unfold protein response (UPR) and activates the ER-stress-induced apoptotic pathway [330]. There is additional evidence implicating ER stress in the neuronal death that occurs in AD [331, 332]. Here, we aimed to address whether the N-Aβcore can protect against Aβ-induced ER stress. We examined a protein kinase-like endoplasmic reticulum kinase (PERK) as an ER stress marker. Activation of PERK via phosphorylation couples ER stress to inhibition of protein translation [333]. Treatment with nM Aβ activates PERK in α4β2-nAChR-transfected cells (Fig. 22). Additionally, in preliminary experiments pM-nM N-Aβcore reduced Aβ activation of PERK (Fig. 22), suggesting that the N-Aβcore can protect against Aβ-induced ER stress.

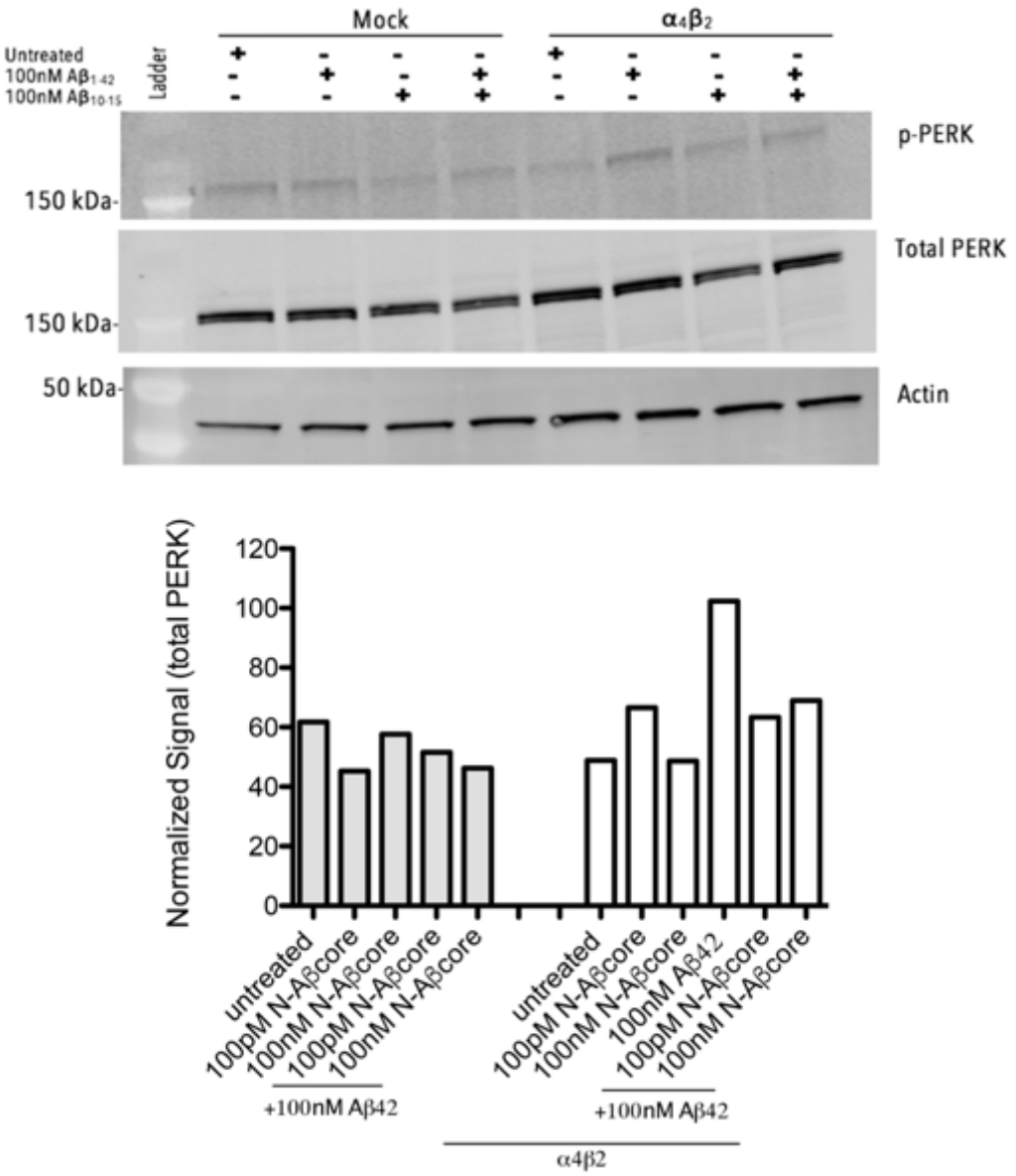


Figure 22: Aβ-induced activation of PERK in nACHR-transfected cells

Western blot of PERK in protein extracts from nAChR-expressing NG108-15 cells treated with N-Aβcore, Aβ or both. (Top) Representative western immunoblot image showing levels of pPERK, total PERK, and control actin. (Bottom) Quantification of the expression of pPERK normalized to total PERK. Treatment concentrations were 100pM or 100nM. n=2, n refers to number of replicates.

3.4 Discussion

Previous studies have established a strong link between the progression of AD and the extent of synaptic dysfunction occurring in the early stages of the disease, prior to the formation of Aβ plaques and tau neurofibrillary tangles [302]. While the exact mechanism of synaptic dysfunction and eventual loss is not well understood, evidence suggests that pathological levels of Aβ induces NMDA receptor-linked excitotoxicity, and this over-stimulation of NMDA receptors activates a cascade of pathological signaling which accounts for neuronal death and synaptic dysfunction [28, 29, 306] in AD.

3.4.1 Impact of Aβ on LTP vs. LTD

Two key mechanisms underlying synaptic plasticity and memory formation are LTP and LTD induction. Although it is well determined that detrimental levels of soluble Aβ have been implicated in LTP inhibition [20, 303, 304], the link between pathological levels of Aβ and LTD are less understood, even showing conflicting results. Alternatively, very low levels (pM) of soluble Aβ have been shown to enhance synaptic plasticity and facilitate hippocampal-based learning and memory [32], suggesting a neuromodulatory role of soluble Aβ at physiological levels. In addition, similar results using an N-terminal fragment of Aβ (1-15) implicated that sequence within Aβ as accountable for the positive neuromodulatory activity of full-length Aβ [62]. Thus, we wondered whether the N-Aβcore (10-15), encompassing the essential sequence within the N-terminal fragment, and be inference, Aβ for the positive neuromodulatory activity could enhance synaptic plasticity and protect against Aβ-induced synaptic dysfunction.

In accordance with previous findings [327], we found that pathological levels of endogenous soluble Aβ significantly inhibits LTP induction, but treatment with the N-Aβcore reversed this deficit. Although not significant, treatment with the N-Aβcore in wild-type slices showed a trend towards LTP enhancement. This suggests that the protection against Aβ-induced LTP deficits by the N-Aβcore is not solely due to competitive binding for target receptors, but may also be in combination with the activation of a neuroprotective pathway that enhances synaptic plasticity. The exact pathway that is activated is not yet known, but we suspect that key players involved in synaptic modulation are affected, such as upregulating CREB, PKA, and/or CAMKII or downregulating calcineurin and/or PP1, subsequently increasing AMPA receptor trafficking to the synapses [334, 335]. Additionally, the enhancement of the basal synaptic transmission with the treatment of the N-Aβcore in both 5xFAD and B6.SJL slices suggests a receptor-linked influx of Ca^{2+}, which further supports the idea that the N-Aβcore activates an alternative neuroprotective pathway that enhances synaptic plasticity. Previously, it has been shown that BDNF enhances basal synaptic transmission [336], therefore, we suspect that the N-Aβcore may be upregulating BDNF expression, possibly through the increase in CREB activation and/or expression [148, 216]. Another possibility is that the N-Aβcore-induced Ca^{2+} influx could also be responsible for BDNF release at the synapses, thus, enhancing baseline synaptic transmission and ultimately LTP. It would be interesting to examine the effect of the basal synaptic transmission by the N-Aβcore long-term to assess whether this is a transient or permanent increase, and whether the enhancement of LTP observed was due to the changes in baseline transmission.

Without a proper mechanism to selectively weaken the synapse after strengthening caused by LTP, no new information would be encoded due to a ceiling-effect, and thus, LTD is necessary for neural homeostasis. To date, there are conflicting studies about the effects of pathological Aβ on LTD, where some groups show that synthetic Aβ enhances LTD [306, 328, 337, 338] and others show no effect [329, 339]. Here, we found that the presence of endogenous soluble Aβ, shown to be present in the brains of 5xFAD mice, resulted enhances LTD in isolated hippocampal slices, and treatment with the N-Aβcore prior to and during the LFS induction of LTD reverses this enhancement. Interestingly, Hu *et. al*. found that applying synthetic soluble Aβ prior to LFS did not affect the early phase of LFS-induced LTD (<2h post LFS), but facilitated the late phase (3-5 h post LFS) [337], thus, possibly accounting for the reason why some groups did not report a difference. It is important to note that late phase LTP and LTD require new protein synthesis. Considering the fact that LTD and LTP work in concert to allow for reversible synaptic plasticity, the LFS-induced enhancement of LTD in the 5xFAD slices could affect subsequent LTP, and this may be another reason why an LTP deficit was observed in the 5xFAD slices compared to wild-type.

While, as noted, NMDA and AMPA receptors are involved in different aspects of LTP and LTD, and are affected by elevated Aβ, metabotropic glutamate receptors (mGluRs) have also been implicated in Aβ-induced synaptic dysfunction [244, 306, 338]. It would therefore be interesting to further address the role of mGluRs in Aβ-linked synaptic deficits and the reversal these deficits by the N-Aβcore.

3.4.2 Intracellullar signaling pathways in Aβ-induced neurotoxicity and synaptic dysfunction

Turning to intracellular signaling pathways, the activation of the MAPK pathway has been strongly implicated in Aβ-induced neurotoxicity and synaptic dysfunction [244, 256, 340]. Specifically, we have shown that treatment with Aβ in the $\alpha4\beta2$-nAChR-transfected NG108-15 cells increased ERK and JNK activation (via phosphorylation) in the early stages of the apoptotic process, thus contributing to the Aβ-induced neuronal toxicity in our model [136]. Taken together with the fact that the N-Aβcore has been shown to protect against Aβ-induced apoptosis and synaptic deficits, we aimed to better understand the neuroprotective mechanism of the N-terminal fragments against Aβ-toxicity, starting with its effect on the MAPK pathway.

In accordance with our previous findings, Aβ increases activation of both ERK and JNK in the presence [136] and absence of the sensitizing nAChRs. However, the reduction of Aβ activation of ERK by the N-Aβcore was found to be nAChR-dependent. This does not come at a complete surprise considering the evidence linking nAChRs to ERK activation [341–344] and downstream of that, CREB phosphorylation and translocation [344]. Thus, we postulate that the ERK-linked synaptic enhancement of the N-Aβcore is mediated by the nAChRs.

On the other hand, the reduction of Aβ-induced JNK activation by the N-Aβcore was found to be nAChR-independent, and more importantly, only pM concentration of the N-Aβcore inhibits JNK activation. These data suggest that at lower concentrations the N-Aβcore blocks apoptosis by inhibiting JNK activation. The exact mechanism of this inhibition is currently not understood but we postulate that the N-Aβcore may upregulate

a JNK-specific phosphatase or inhibit JNK phosphorylation by modulating one of the scaffolding proteins. Furthermore, studies have shown that JNK1-deficient models have enhanced memory [345, 346], and therefore, we speculate that the inhibitory influence of the N-Aβcore on JNK phosphorylation contribute to the N-Aβcore LTP enhancement and protection against Aβ-induced LTP deficits. The fact that this degree of inhibition was only observed at pM levels of the peptide suggests that high concentrations (nM) of the N-Aβcore may somehow be masking this action of pM N-Aβcore. Moreover, the reduction in oxidative stress to below baseline levels and upregulation of CREB activity and expression observed with pM N-Aβcore further supports our postulate that the N-Aβcore is eliciting an alternative neuroprotective pathway, and although still protective, nM concentrations of the N-Aβcore may be attenuating the effects seen at pM concentrations.

Taken together, our results suggest a concentration-dependent neuroprotective action of the N-Aβcore on synaptic plasticity. While the exact molecular pathway still remains to be elucidated, we postulate that the N-Aβcore binds to target receptors to modulate the expression of proteins involved in enhancing synaptic plasticity.

CHAPTER 4: CONCLUSION

4.1 Final Remarks

A tremendous amount of effort in both scientific and clinical research has been focused on understanding and developing a cure for AD. Unfortunately, many of the potential therapeutic agents have fallen short in clinical trials, resulting in only two classes of FDA-approved drugs for AD treatment on the market. This is in part, due to the fact that AD is a multifactorial disorder that involves multiple biological pathways, and thus, targeting one pathway for treatment has proved to be unsuccessful. In addition, soluble Aβ deposition and Aβ-toxicity occurs years before the onset of AD symptoms [278]. Therefore, it is imperative to begin treatment at the right window of opportunity to halt the progression of the disease and to preserve any existing neuronal and synaptic function. Furthermore, a disruption in Aβ's normal physiological function could contribute to further toxicity [36].

Previous studies have found Aβ monomers to be protective against oligomeric Aβ toxicity [128], but maintaining stable monomeric Aβ *in vivo* may be hard, if not impossible to achieve. Alternatively, an N-terminal fragment of Aβ (1-15/16) naturally derived from Aβ by the action of α-secretase, which does not oligomerize, is not toxic and whose sequence was shown to be responsible for the neuromodulatory activity of full-length Aβ [62], may provide an entirely different means to approach AD therapy. Here, we showed that a core hexapeptide sequence within this fragment (N-Aβcore), encompassing the key residues attributing to the neuromodulatory activity to the N-terminal fragment, was able to protect against Aβ-induced mitochondrial dysfunction, oxidative stress and apoptotic neuronal death, whether added as co-treatment or post Aβ treatment, as well

as rescue Aβ-induced LTP and LTD deficits in 5X FAD model mice. It was further found that the N-Aβcore protects against Aβ toxicity involving high affinity Aβ receptors (nAChRs) as well as other low affinity Aβ target receptor(s), suggesting that the N-Aβcore may be broadly neuroprotective through altered interaction of Aβ with all key target sites and/or activation of alternative pathways, such as the aforementioned neuroprotective pathways, making it a highly effective neuroprotective agent against Aβ-linked neuronal toxicity, synaptic dysfunction, and behavioral deficits. Moreover, the peptide is small enough to provide a platform for small molecule peptidomimetic drug development.

The precise mechanism by which the N-Aβcore exerts its effect(s) is still not fully understood, but from our findings, we postulate that the N-Aβcore acts as a competitive (partial) agonist binding to target receptors to (1) modulate Ca^{2+}-dependent pathways, (2) the MAPK pathway and (3) elicit a signaling cascade to upregulate proteins to enhance cell survival and synaptic plasticity. Direct demonstration that the N-Aβcore competitively blocks Aβ binding target receptors via ligand binding assays remains to be determined. However, we have previously shown that concurrent administration of both nanomolar N-Aβ fragment and micromolar Aβ to cells expressing nAChRs resulted in a decrease in the Ca^{2+} response compared to Aβ alone [62], suggesting competition between the two. Here, we showed that late addition of the N-Aβcore after initiating Ca^{2+} responses with either the N-Aβ fragment or Aβ alone also sharply reduced the responses. Therefore, we suspect the N-Aβcore acts as a partial agonist to competitively antagonize Aβ binding to target receptors, attenuating Aβ-induced toxicity, and thus, partially contributing to the neuroprotection observed. On the other hand, our results indicate that the N-Aβcore is

also elicits an alternative protective pathway that attenuates and/or inhibits Aβ toxicity (Fig. 23).

Taken together, our data suggest that the small, nontoxic N-Aβcore not only protects against Aβ-induced neuronal and synaptic toxicity, but when administered at the later stages of the disease, would be capable of preserving cellular and synaptic function. In addition, we have shown that stabilization of the N-Aβcore retains functionality, therefore, providing a good platform for further drug development.

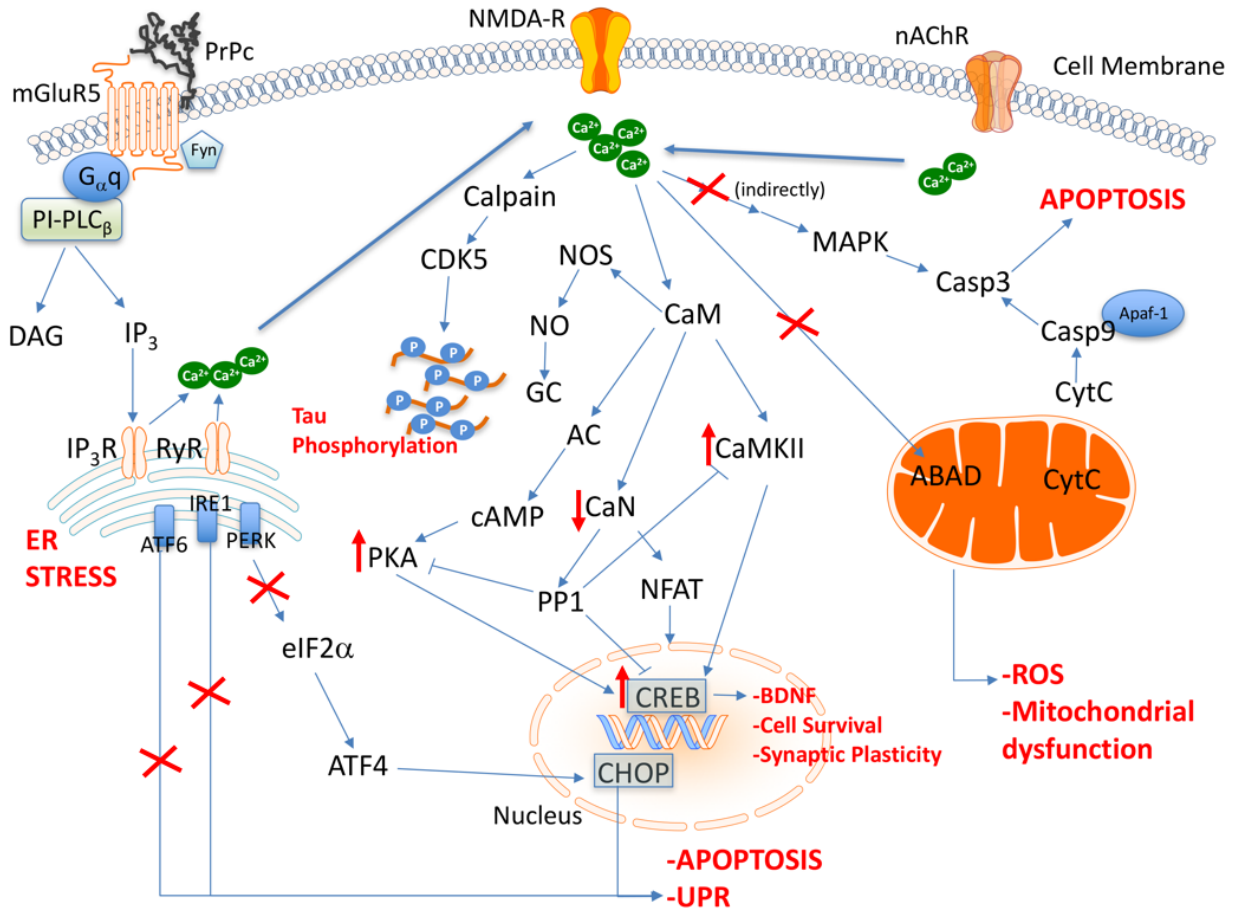


Figure 23: Suggested neuroprotective action of the N-Aβcore against Aβ toxicity

Although the exact pathway is still not fully identified, the N-Aβcore has been shown to protect against Aβ-induced ER-stress, mitochondrial dysfunction, oxidative stress and MAPK-linked apoptosis. The synaptic enhancement by the N-Aβcore may be attributed to an increase in PKA and CaMKII activity, and thus, increasing CREB activity and possibly expression.

4.2 Future Directions

It is well established that Aβ interacts with numerous receptors [141]. In addition to the high-affinity nAChRs, two prominent targets linked to Aβ are the cellular prion protein (PrPc) and paired immunoglobulin-like receptor B (PirB) [347]. It will be important to address the specific role(s) for PrPc and/or PirB in the action of the N-Aβcore to protect against Aβ neurotoxicity and synaptic and behavioral dysfunction.

An intriguing finding in our studies is that pM concentrations of the N-Aβcore shows the most robust effect in reducing oxidative stress, blocking JNK activation and upregulating CREB activity and expression, indicating a highly potent action of the N-Aβcore peptide. We have postulated that nM concentrations of the N-Aβcore, although still protective, attenuate or outright reverse the response at pM levels, and therefore, further studies identifying the exact molecular pathway(s) regulated by pM N-Aβcore is essential. A good starting point would be to perform RNA-sequencing to assess which proteins are affected with pM as compared to nM N-Aβcore treatment.

Another interesting result was the increase in basal synaptic transmission with the treatment of the N-Aβcore. It would be interesting to assess whether this increase is sustained over a long period of time or if it falls back to baseline levels. Additionally, elucidating the underlying mechanism of the N-Aβcore may also speak to this observed enhancement. For example, CREB has been shown to upregulate BDNF, an important regulator in synaptic remodeling and cognitive processes [148, 216], and therefore, the upregulation of CREB activity and expression in our neuronal model may contribute to the synaptic enhancement.

Additionally, more in-depth electrophysiology experiments should be performed to address the exact contribution of the NMDA, AMPA and metabotropic glutamate receptors in the impact of the N-Aβcore as well as Aβ regulation LTP and LTD induction and expression. In addition to the aforementioned receptors, the role of PrPc should also be explored in the neuroprotective action of N-Aβcore on synaptic plasticity.

In this study, we did not look at the effects of the N-Aβcore on tau phosphorylation. An interesting upstream Ca^{2+}-dependent regulator of tau phosphorylation is calpain. Activation of calpain increases the activity of several kinases implicated in phosphorylating tau, such as cyclin-dependent kinase 5 (CDK5), glycogen synthase kinase β (GSKβ), and MAPK [348]. Thus, assessing the activity of calpain and the aforementioned kinases with N-Aβcore treatment would be of interest.

Lastly, a more physiological relevant model will need to be studied to substantiate our findings. In addition to our neuronal model, it would be important to confirm the molecular pathway regulated by the N-Aβcore in primary hippocampal cultures and/or hippocampal slice cultures. Furthermore, analysis on the hippocampal slices after LTP or LTD induction to assess changes in expression of key proteins implicated in synaptic plasticity and neuroprotection should be explored. Overall, these studies will address the neuroprotective mechanisms of the N-Aβcore on neuronal survival and synaptic modulation.

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